The effects of temperature and ploidy on the metabolism and energetics of Atlantic salmon (*Salmo salar*) infected with amoebic gill disease

by Alyssa J. Bowden

Bachelors of Science (University of North Carolina at Wilmington)

Masters of Applied Science (James Cook University, Townsville)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

at the

Institute for Marine and Antarctic Studies

University of Tasmania, Australia

in collaboration with the

Commonwealth Scientific and Industrial Research Organisation

January 2018

Declarations and Statements

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Authority of Access

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time, the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Signed: _____

Alyssa J. Bowden, Candidate

Date: 12 January 2018

Statement of Publication

Original research papers in peer-reviewed journals

Bowden, A.J., Andrewartha, S.J., Elliott, N.G., Frappell, P.B., Clark, T.D. (2018). Negligible differences in metabolism and thermal tolerance between diploid and triploid Atlantic salmon (*Salmo salar* L.). *Journal of Experimental Biology*. Jeb-166975.

Oral presentations

Bowden, A.J., Elliott, N., Frappell, P., Andrewartha, S.J., Clark, T.D. (2016) Late-progression amoebic gill disease impairs thermal tolerance in infected Atlantic salmon. Presentation at the Society for Experiment Biology Annual Meeting, Brighton, U.K.

Bowden, A.J., Maynard, B., Morash, A., Elliott, N., Frappell, P., Andrewartha, S.J. (2014) Do Atlantic salmon and brown trout hybrids have superior metabolic physiology and swimming performance? Presentation at the Australia and New Zealand Society for Comparative Physiology and Biochemistry, Armidale, NSW.

Poster presentations

Bowden, A.J., Elliott, N., Frappell, P., Andrewartha, S.J., Clark, T.D. (2016) Do the energetics and thermal tolerance of triploid and diploid Atlantic salmon differ? Presentation at Species on the Move, Hobart, TAS.

Statement regarding published work contained in thesis

The publishers of the papers comprising Chapter 2 hold the copyright for that content and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Signed: _____

Alyssa J. Bowden, Candidate

Date: 12 January 2018

Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Candidate: Alyssa J. Bowden, IMAS- University of Tasmania and CSIRO, Hobart, Australia

Author 1: Timothy D. Clark, IMAS- University of Tasmania and CSIRO, Hobart, Australia, Primary supervisor

Author 2: Sarah J. Andrewartha, CSIRO, Hobart, Australia, Supervisor

Author 3: Nicholas G. Elliott, IMAS- University of Tasmania and CSIRO, Hobart, Australia, Supervisor

Author 4: Peter B. Frappell, IMAS- University of Tasmania, Hobart, Australia, Supervisor

Author details and their roles:

Paper 1 (Located in Chapter 2): Negligible differences in metabolism and thermal tolerance between diploid and triploid Atlantic salmon (*Salmo salar* L.)

Candidate developed the research idea, conducted the experiment, collected and analysed the data and wrote the manuscript (80%). Authors 1, 2, 3, and 4 assisted with project idea development, data interpretation, and revising the manuscript.

Signed: _____

Alyssa J. Bowden, Candidate

Date: 09 Jan 2018

Supervisor and Head of School Declaration

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted peer-reviewed manuscripts contributing to this thesis:

Signed: _

Dr. Timothy D. Clark
Supervisor
IMAS
University of Tasmania

Date: 4 Jan 2018

Prof. Chris G. Carter (PhD)
Head of School
IMAS
University of Tasmania

8 Jan 2018

Acknowledgements

The past four years have been a wonderful experience that I could not have completed without the guidance of my amazing supervisors Dr. Timothy D. Clark, Dr. Sarah J. Andrewartha, Dr. Nick G. Elliott, Dr. Peter B. Frappell, and Dr. Andrea J. Morash. I thank all of you for the support, encouragement, explanations, countless edits, and endless patience with my numerous questions.

I am extremely grateful for financial help and support from the Sense-T program and the Australian Research Training Program scholarship. I would also like to thank the University of Tasmania, the Institute of Marine and Antarctic Studies, the Society of Experimental Biology, and the Australian and New Zealand Society of Comparative Biochemistry and Physiology for financial support in conference travel.

To my fellow PhD candidates: Andrew Wood and Katharina Alter, thank you for the coffee runs, office discussions and laughs, help during experiments, and most importantly emotional and mental support as we went through the process together. A special thanks to my colleagues from CSIRO as well. Thank you to Richard Taylor and Ben Maynard for introducing me to the practical side of aquaculture research and their invaluable help in learning about amoebic gill disease. To Harry King, Peter Kube, John McCulloch, Matthew Hamilton, Dave Cordell and Elias Polymereropoulos, thank you for taking a keen interest in my research and for the support throughout my candidature. Thank you to Melissa Humphries for statistical help and support.

The farmhands, managers, and technical support I have had also deserve a special thanks to make this thesis possible. Thank you to Mark Hilder and Brad Evans for help in sourcing fish for my experiments. To Joel Slinger and David Blythe from the Bribie Island Research Centre, thank you for the invaluable help in making Chapter 3 possible through fish sourcing, infection, and experimental set-up. Thank you to Mark Adams and Hendrick from the Launceston Aquaculture Centre for the technical support, experimental set-up, and invaluable assistance for Chapter 4.

To my family, thank you so much for your unconditional love and support throughout my PhD and supporting my intercontinental move.

To my friends, both here and in the States, thank you for your continued support and always being there when I needed someone to lean on.

General abstract

Atlantic salmon (*Salmo salar*) aquaculture is an important industry from the global down to local markets. In Tasmania, the industry faces a serious health risk in the form of amoebic gill disease (AGD). The disease attributes 14 to 20% of production costs through control measures and mortalities. The warmer summer months result in proliferation of AGD suggesting that the 1.3 to 3˚C temperature increases predicted by the end of the century could detrimentally impact the Atlantic salmon aquaculture industry. This thesis investigates current and future temperature scenarios on chronic and acute thermal tolerance of aquaculture-relevant species and disease status. Specifically, the focus is on respiratory physiology under potentially stressful environmental conditions.

The production of triploids can be advantageous to the aquaculture industry due to their inherent sterility allowing them to reach market size without the stress of maturation. In addition, triploids present a unique experimental model to investigate physiological processes due to their altered genome (e.g. larger but fewer cells). Despite observations of reduced thermal tolerance in triploids compared to their diploid counterparts, negligible differences in metabolism or thermal tolerance were found between ploidies in Chapter 2. Diploid and triploid Atlantic salmon were acclimated to three temperatures (10, 14, and 18°C) at which their metabolic rates (resting and maximum) and acute thermal tolerance was determined. The experiment was conducted over 9 weeks with measurements occurring at weeks 0 (mass), 3 (mass and metabolic rates), 7 (mass and metabolic rates), and 9 (mass, metabolic rates, and critical thermal maximum [CT_{max}]). While mass, specific growth rate (SGR), and resting metabolic rate ($\dot{M}O_{2rest}$) were significantly different in the beginning weeks of the experiment, all three converged by week 7 of the experiment. Maximum metabolic rate ($\dot{M}O_{2max}$), and aerobic scope ($\dot{M}O_{2max}$- $\dot{M}O_{2rest}$) remained stable across acclimation temperatures, measuring time points and ploidy. Furthermore, CT_{max} was found to be independent of ploidy. This study suggests that triploidy does not inhibit thermal tolerance in juvenile Atlantic salmon, so therefore diploids were utilized in subsequent chapters.

Amoebic gill disease attaches solely to the gills and causes proliferation of the gill epithelium resulting in fusion of the secondary lamellae. This potentially reduces the functional surface area for oxygen uptake. Furthermore, the disease could have adverse effects on the host during periods of poor environmental conditions such as elevated temperatures or hypoxia.

Across two chapters, the thermal tolerance and metabolism of AGD-infected diploid Atlantic salmon was investigated. Severely infected Atlantic salmon had impaired acute thermal tolerance as evidenced by a decreased CT_{max} temperature in Chapter 3. In Chapter 4, naïve and AGD-infected Atlantic salmon were acclimated to 15 and 19°C and $\dot{M}O_{2rest}$, $\dot{M}O_{2max}$, aerobic scope, excess post-exercise oxygen consumption (EPOC), and hypoxia tolerance (P_{crit}) were assessed. Increasing infection level was positively correlated with $\dot{M}O_{2rest}$ at both acclimation temperatures while $\dot{M}O_{2max}$ remained stable. The increase in $\dot{M}O_{2rest}$ without a concurrent increase in $\dot{M}O_{2max}$ caused aerobic scope to decrease with increasing infection level. Furthermore, evidence was found for impaired hypoxia tolerance. These findings suggest that heatwaves and periods of hypoxia could be detrimental to AGD-infected salmon.

This thesis demonstrates that future climate change scenarios could have an impact on the Atlantic salmon aquaculture industry. It concludes that the effects of AGD on Atlantic salmon impairs acute thermal tolerance which could be detrimental with the projected increase in prevalence of heatwaves with climate change. However, given the chance for acclimation (i.e. an increase in average temperatures), infected salmon at higher temperatures (e.g. 19°C) could cope as well as those at lower acclimation temperatures (15°C).

Table of Contents

List of Figures and Tables

Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix A

Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abbreviations

AGD	Amoebic gill disease
CL-T	Chloramine-T
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT_{max}	Critical thermal maximum
EPOC	Excess post-exercise oxygen consumption
[Hb]	Haemoglobin concentration
Hct	Haematocrit
LOE	Loss of equilibrium
MCHC	Mean corpuscular haemoglobin concentration
$\dot{M}O_2$	Oxygen uptake rate
$\dot{M}O_{2max}$	Maximum oxygen uptake rate
$\dot{M}O_{2rest}$	Resting oxygen uptake rate
OCLTT	Oxygen- and capacity- limited thermal tolerance
P_{CO2}	Partial pressure of carbon dioxide
P_{crit}	Critical oxygen concentration
P_{O2}	Partial pressure of oxygen
SGR	Specific growth rate
T_{opt}	Optimum temperature
U_{crit}	Critical swimming speed

Chapter 1: General Introduction

Climate warming

Global trends

The greenhouse gas theory dates back more than 150 years (Tyndall, 1863; Arrhenius, 1896), yet the effect of climate change on the biology of animals is an increasingly prominent area of research. Current near-term climate change models predict an increase in mean air temperature of 0.3 to 0.7°C by 2035 and longer-term increases of 1.0 to 3.7°C by 2100 (Collins et al., 2013a; Kirtman et al., 2013). While it is likely that temperatures will increase to a greater extent over land masses compared to the oceans, projected increases in sea surface temperature range from 1.0 to 3.0˚C by the end of the century and the additional heat is expected to reach a depth of 1 km (Collins et al., 2013a). Furthermore, the frequency and intensity of heatwaves are predicted to increase substantially throughout this century (Kirtman et al., 2013), and there are documented 'hotspots' like south-eastern Australia where heatwaves and the magnitude of warming are substantially greater than the global average (Frusher et al., 2013). Therefore, understanding the impact of chronic and acute temperature elevations on individuals, populations, and communities, particularly in warming hotspots, is critical to forecast the near-future effects of climate change on animal biodiversity.

Elevated temperatures are expected to affect species at tropical latitudes to a greater extent than those in more temperate habitats (Tewksbury et al., 2008). This could either be because the former evolved in stable thermal environments resulting in narrower thermal tolerance ranges (Tewksbury et al., 2008), or because biological processes increase exponentially with temperature resulting in proportionately faster (and detrimental) rates in tropical systems (Payne and Smith, 2017). At similar latitudes, seasonal temperature variability is suppressed in aquatic environments compared to terrestrial habitats as water has a large heat storage capacity (Sunday et al., 2011), meaning aquatic animals have evolved in more thermally stable environments than terrestrial organisms of the same latitudes. However, the effect of climate change across latitudes not only depends on the magnitude of the temperature shift, but also on the behaviour, morphology, physiology, and ecology of the species in question (Kearney and Porter, 2004; Helmuth et al., 2005; Bradshaw and Holzapfel, 2008).

Effects on ectotherms

Temperature is a key environmental factor influencing the performance and fitness of ectotherms/poikilotherms due to their inability to physiologically thermoregulate (Deutsch et al., 2008). Species inhabiting broader, more thermally variable habitats are suggested to exhibit greater tolerance to acute temperature increases compared to those that inhabit narrower, more thermally stable environments (Magozzi and Calosi, 2015). Furthermore, while species acclimated to higher temperatures achieve a higher critical thermal maximum (CT_{max}), their warming tolerance (CT_{max} minus acclimation temperature) is reduced compared to cooler acclimated species, consequently reducing their buffering capacity to cope with acute thermal stress events (Deutsch et al., 2008; Tewksbury et al., 2008). For example, when Sandblom et al. (2016) compared European perch (*Perca fluviatilis*, L.) from a natural thermal regime with individuals that had experienced chronically warmer conditions over three decades (5 to 10°C warmer due to effluent water from a nuclear power plant), they found a significantly higher CT_{max} in the chronically warmer fish (by 2.2°C), but the warming tolerance was significantly lower (Δ4.6°C compared to Δ10.1°C in control fish). In light of the continuous warming of global average temperatures and the increased incidence of heatwaves, it is important to understand the capacity of ectothermic animals to respond and survive.

In this context, aerobic metabolism has been termed the 'fire of life' and is intimately dependent on temperature (Kleiber, 1961). The internal body temperature of fish, being ectotherms, is normally within a few fractions of a degree of the surrounding water (Wood and McDonald, 1997). Therefore, higher temperatures increase the energy requirements for basal metabolic processes of ectotherms, subsequently reducing the energy that can be allocated to key life-history processes such as growth, reproduction, and foraging (Brett, 1971). The importance of aerobic metabolism has led to it being implicated as the underlying mechanism driving the responses of species to climate change, in accordance with the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (Fry, 1947; Claireaux and Lefrançois, 2007; Pörtner and Knust, 2007).

The OCLTT hypothesis focuses on aerobic scope (maximum metabolic rate [$\dot{M}O_{2max}$] minus resting metabolic rate [$\dot{M}O_{2rest}$]), which represents the capacity to increase oxygen uptake rate (and aerobic energy production) above resting levels. Thus, aerobic scope theoretically represents the capacity of an animal to simultaneously supply energy to processes such as

growth, locomotion, and reproduction (Pörtner et al., 2001; Claireaux and Lefrançois, 2007). The OCLTT hypothesis postulates that there is an optimum temperature for aerobic scope ($T_{opt(AS)}$) that coincides with the temperature enabling peak fitness-related processes (e.g. growth, reproduction, and locomotion) (Fig. 1.1A). On either side of $T_{opt(AS)}$, aerobic scope is postulated to decrease, diminishing fitness-related performance traits and characterising the thermal tolerance window (Frederich and Pörtner, 2000; Pörtner et al., 2001). In addition, thermal windows are proposed to become narrower with synergistic stressors (e.g. hypoxia, ocean acidification) causing a lower aerobic scope at $T_{opt(AS)}$ and a lower maximum critical temperature (Fig. 1.1A) (Pörtner and Farrell, 2008). However, the OCLTT hypothesis has generated vociferous debate because many species have been found to have thermally-independent aerobic scope or a continuous increase in aerobic scope up to maximum critical temperatures (Clark et al., 2011; Norin et al., 2014). The debate was summarised in Clark et al. (2013), and an alternative schematic was presented that showed aerobic scope increasing past the optimal temperature for performance (e.g. growth and fitness) and declining abruptly immediately prior to the upper critical temperature (Fig. 1.1B).

Regardless of any changes in aerobic scope that may occur with an increase in temperature, the increase in basal energy/oxygen requirements of ectotherms is concomitant with a decrease in water oxygen (O_2) solubility, resulting in less O_2 available to supply the elevated metabolism. Therefore, ectotherms have to either alter or acclimate their behaviour or physiology to increase O_2 uptake to cope with elevated temperatures. Some wild populations of fish undergo shifts in biogeographical ranges (primarily towards the poles) to find more suitable thermal environments (Parmesan and Yohe, 2003; Perry et al., 2005; Parmesan, 2006), but it is not always an option for fish to move. One example of the latter scenario is in aquaculture, where fish that cannot relocate to cooler water must enhance their oxygen transport potential (through behavioural or physiological acclimation) or suffer mortality.

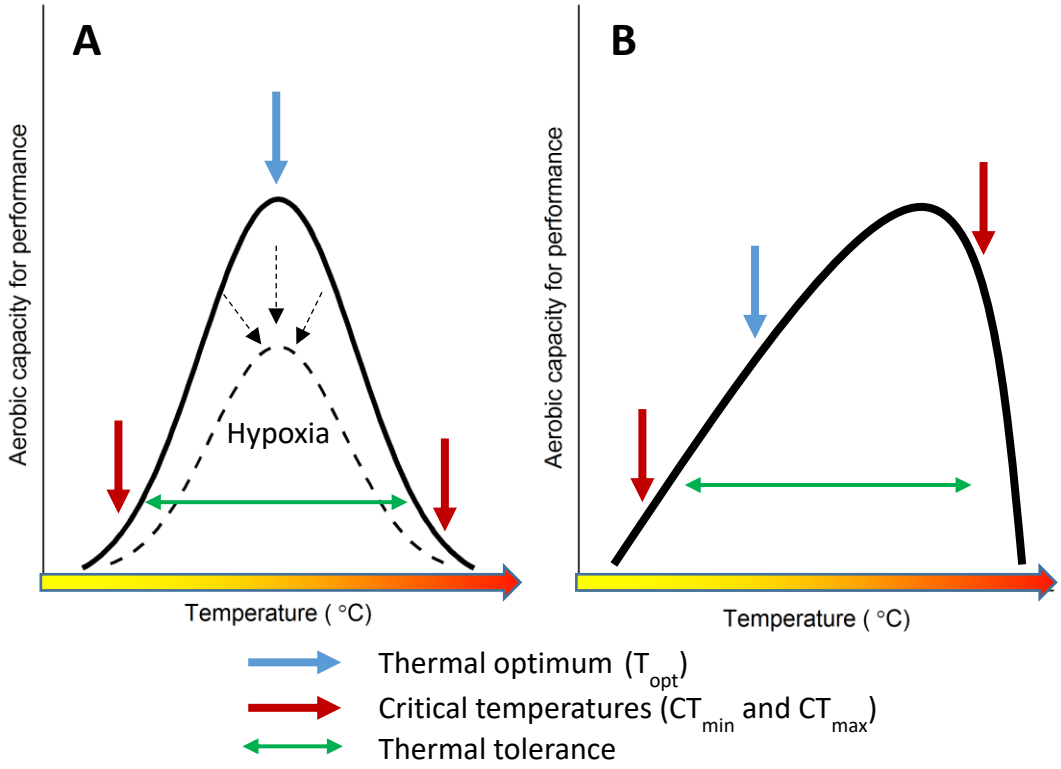

Figure 1.1: Hypothetical schematics depicting (A) a proposed thermal window based upon the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (modified from Pörtner and Farrell (2008)) and (B) an alternative explanation for how aerobic performance aligns with the optimal temperature of the organism (blue arrow) and responds to an increase in temperature (modified from Clark et al. (2013)). The primary difference being that A assumes that the thermal optimum coincides with maximal aerobic scope, whereas aerobic scope continues to increase in B past the animal's thermal optimum, and decreases rapidly immediately prior to the upper critical temperature. Also depicted in A is the hypothetical impact of an additional stressor (e.g. hypoxia, CO_2) on aerobic scope and the breadth of the thermal window.

Effects on aquaculture

Aquaculture can present a unique challenge to fish in the context of climate warming, since the stock are restricted to holding units, such as cages/pens, and cannot migrate to cooler or more suitable water. Within cages/pens, the salmon behaviourally select preferred conditions in the water column through active avoidance of low dissolved oxygen (<35% saturation) and warm surface waters (>20.1°C) (Stehfest et al., 2017). These behaviours vertically contract the water column inhabited within sea cages which could become exacerbated with increasing

temperatures with climate change. Aquaculture species are almost exclusively ectothermic, so their inherent physiology makes them more susceptible to the increase in temperature than their terrestrial agricultural counterparts that are typically endothermic (Buckley et al., 2012). The temperature-related increase in basal metabolic processes of ectotherms has the potential to decrease the production volume of the industry (Cochrane et al., 2009). Moreover, the observed increase in heatwaves could be detrimental to the aquaculture industry if the magnitude of change in temperature exceeds the thermal tolerance range of the species. Even sub-lethal temperature increases can cause major production losses, due to a breakdown of homeostasis in biochemical and physiological processes.

Global warming can also impact aquaculture stocks indirectly through diseases. Elevated temperatures can benefit pathogen viability, disease transmission and host vulnerability, although a subset of pathogens may suffer at higher temperatures and release their hosts from the threat of disease (Harvell et al., 2002). Marine diseases have started appearing in areas where they were previously unseen as a result of either hosts or pathogens expanding their ranges, often in response to global warming (Cochrane et al., 2009). Notably, an east coast oyster disease (*Perkinsus marinus*) in the U.S. expanded its range from Long Island to Maine during a winter warming trend when cold waters would typically inhibit pathogen growth (Ford, 1996; Cook et al., 1998). Disease issues constitute the largest economic losses in aquaculture (Meyer, 1991), so an increase in disease episodes due to global warming could be disastrous for such industries.

Understanding the effect of both the direct and indirect stressors of climate change on aquaculture species is important to help ensure sustainable farming practices in the future. Locations that are currently suitable for aquaculture may become unsuitable in the years to come and vice versa. Furthermore, farms must be knowledgeable of the effects of environmental parameters on their stock when considering expansion into new areas.

Atlantic salmon aquaculture

Aquaculture industries and fisheries play an important role economically and in food supply from global through to local levels. While Oceania is a minor contributor in the global market by producing less than 1% of the seafood value, Australian aquaculture industries contribute just over half of that (~52%) (Fig. 1.2A, B) (FAOSTAT, 2015). Aquaculture production occurs throughout Australia, but is concentrated in regional areas providing jobs and economic growth (ABARES, 2014). The Atlantic salmon (*Salmo salar* Linnaeus) industry

contributes the largest value of production within Australia at ~56%, the majority of which comes from Tasmania (FAOSTAT, 2015) (Fig. 1.2C). The importance of the salmon industry, and aquaculture in general, highlights the need to understand the effects of climate change, particularly in global warming 'hotspots' like south-east Australia, if we are to ensure the sustainability of the industry into the future.

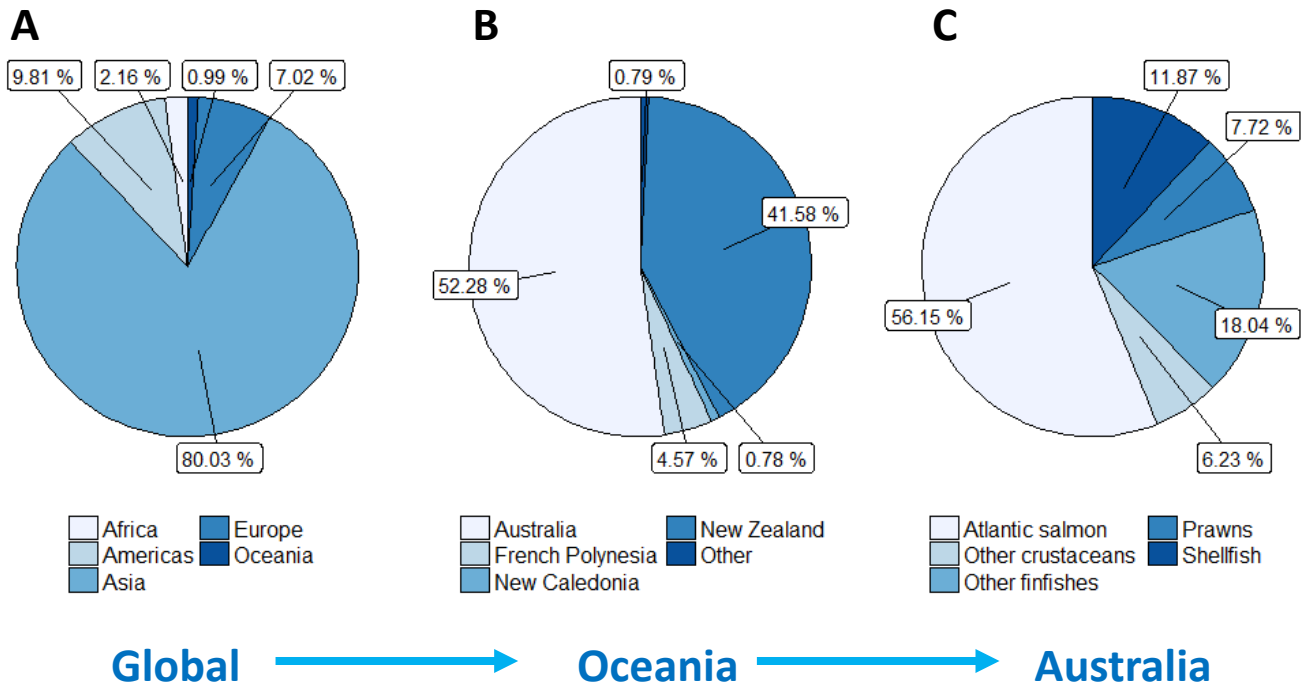

Figure 1.2: Pie charts showing the value contributions of aquaculture to (A) the global market by continent, (B) countries that belong to Oceania, and (C) Australia grouped by species. Data retrieved from (FAOSTAT, 2015) on October 19, 2017.

Atlantic salmon are anadromous so have two phases in which they are cultured: the freshwater and seawater phases. The egg and fry stages (freshwater) occur in inland hatcheries where systems can be in place for managing dissolved gas levels, water temperature and disinfection, and to allow water reuse and the operation of alarm systems (Pennell and McLean, 1996). Once the fish are ponded (parr stage), they are commonly in raceways where environmental conditions may not be controlled. Similarly, once fish are smolted and transferred to seawater cages they are also subject to ambient environmental conditions. Therefore, temperature shifts associated with global warming are likely to impact both stages where the fish are in uncontrolled conditions.

While the lower thermal limit remains similar for Atlantic salmon (0°C) from the egg to alevin to smolt stages, the upper thermal limit (CT_{max}) increases with body size (16, 24 to 25, and 30 to 33°C, respectively) (Grande and Andersen, 1991; Lund et al., 2002; Finstad et al., 2004; Elliott and Elliott, 2010). Within these temperature extremes are optimum temperatures for processes such as growth that are important to aquaculture production. The optimum temperature for Atlantic salmon parr growth is 15 to 19°C which closely matches the average summer sea temperatures off the Tasmanian coast (15 to 17°C) (Elliott and Hurley, 1997; Forseth et al., 2001; Jonsson et al., 2001). As average temperatures increase with global warming, the growth of the Tasmanian stock, and therefore production output, may become compromised.

The Atlantic salmon industry in Tasmania also faces a large health risk to the stock in the form of amoebic gill disease (AGD). The disease was first identified in Tasmania in 1986 (Munday, 1986). Since then, the disease has been reported in a number of other species including rainbow trout (*Oncorhynchus mykiss* Walbaum) in Tasmania (Munday et al., 1990), Ireland (Rodger and McArdle, 1996), and Chile (Bustos et al., 2011), coho salmon (*Oncorhynchus kisutch* Walbaum) in Washington state and California in the U.S. (Kent et al., 1988), turbot (*Scophthalmus maximus* Linnaeus) in Spain (Dyková et al., 1998; Dyková et al., 1999), as well as sea bass (*Dicentrarchus labrax* Linnaeus) in the Mediterranean (Dyková et al., 2000), and brown trout (*Salmo trutta* Linnaeus) in France (Munday et al., 2001). However, of the farmed salmonids, Atlantic salmon are the most susceptible to the disease (Munday et al., 2001), which can lead to death in over 50% of infected individuals (Wallach and Nowak, 2012). It is suggested that the emergence of the disease in some previously disease-free sites is due to an increase in average sea temperatures (Steinum et al., 2008). Indeed, in 1995, clinical signs of AGD were observed on eight Atlantic salmon farms in Ireland when the country experienced the warmest summer sea temperatures ever recorded (Rodger and McArdle, 1996).

Amoebic gill disease

Pathophysiology

The aetiological agent of AGD in Atlantic salmon was previously considered to be solely *Neoparamoeba pemaquidensis* (Kent et al., 1988; Roubal et al., 1989), but later, *N. branchiphila* was also successfully cultured from the gills of AGD-infected fish (Fiala and Dyková, 2003; Dyková et al., 2005). Both were thought to induce AGD due to morphological

similarities with trophozoites associated with AGD lesions (Wong et al., 2004; Dyková et al., 2005). However, using *in situ* hybridisation, Young et al. (2007) observed only one strain directly associated with AGD lesions which belonged to a new phylogenetic lineage called *N. perurans* (now *Paramoeba perurans*). These findings undermined the previously-suggested importance of *N. pemaquidensis* and *N. branchiphila* in AGD infection.

Infected gills exhibit gross signs of slightly raised, white mucous patches (Fig. 1.3A) (Adams and Nowak, 2001). Gross signs do not always match up with histological evidence of disease, which presents itself as hyperplasia (Roubal et al., 1989) and fusion of secondary lamellae (Fig. 1.3B) (Kent et al., 1988; Parsons et al., 2001; Adams and Nowak, 2001; Adams and Nowak, 2003). Hyperplastic lesions vary in size and extent with amoebae often seen in close proximity. While the specific reasoning remains unclear, amoebae are occasionally observed entrapped within interlamellar vesicles or 'cysts' (Kent et al., 1988; Dyková et al., 1995; Parsons et al., 2001), but it has been suggested that the cysts could protect the amoebae from treatment (Parsons et al., 2001).

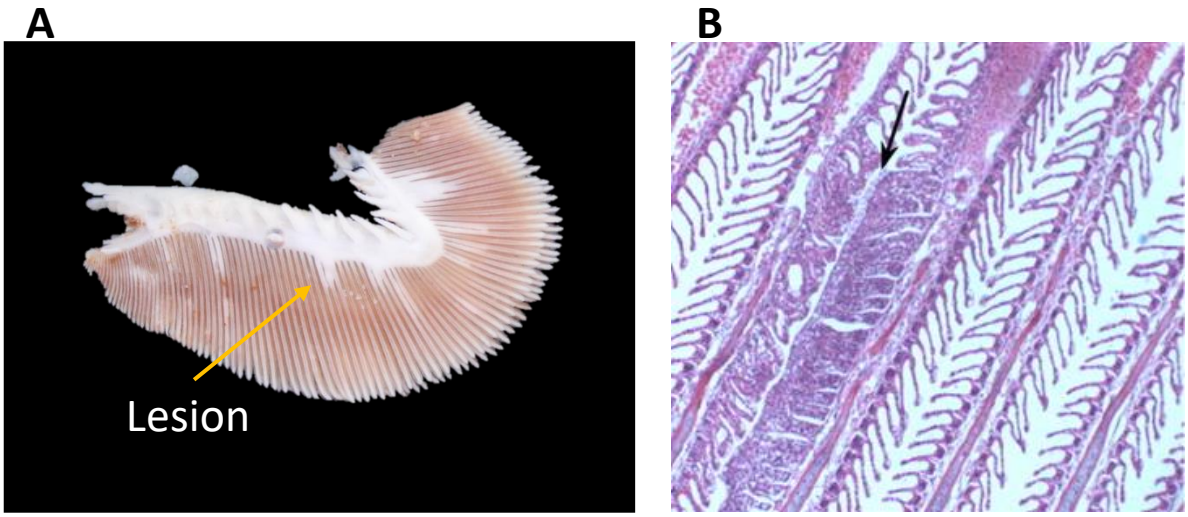

Figure 1.3: (A) Gross view of amoebic gill disease showing the white mucoid patches. The gill was extracted, fixed in seawater Davidson's fixative and then photographed. See Chapter 4 for more details. (B) Histological cross-section of an AGD-infected gill. Note the fusion of the secondary lamellae. The arrow is pointing to amoebae still attached to the gill. Photo was modified from Morrison et al (2006).

Treatment and prevention

Currently, the most effective method to combat AGD is freshwater bathing (Munday et al., 2001; Parsons et al., 2001). The process is an economic burden to farms in labour and infrastructure and is responsible for up to 20% of total production costs (Munday et al., 2001). The need for access to a freshwater source limits the amount of sites suitable for salmon farming. Furthermore, the process is stressful for the stock. Prior to bathing, fish are crowded, and netted out of the pen, anaesthetised, and then 'gill scored'. Gill scoring involves grossly examining all gill arches for AGD lesions and assigning a score based on the percentage covered. Gill scores range from 0 (no lesions present) to 5 (>50% of the gills covered in lesions, Table 1.1) (Taylor et al., 2009a). Once the stock reaches an average score of 3, a freshwater bath is initiated (Taylor et al., 2009a).

Table 1.1: Gill score guide modified from Taylor et al (2009).

Gill score	Infection level	Gross appearance
0	Clear	Gills show no sign of infection and appear healthy
1	Very light	1 white spot or undefined necrotic streaking
2	Light	2 to 3 white spots or mucous patch
3	Moderate	Up to 20% of gill area covered by mucous patches
4	Advanced	Established lesions covering 20 to 50% of gill surface area
5	Heavy	Over 50% of gill area covered by mucous patches

During a bathing event, fish are transferred from their pen into a second pen (usually using a fish pump) which has a tarpaulin liner in it and is filled with freshwater that is oxygenated up to 200% air saturation with a stocking density up to 40 kg m^{-3}. After the last fish is transferred, they remain in the freshwater for 2 to 4 hours before the liner is dropped (Parsons et al., 2001). Clark et al. (1999) showed that freshwater bathing can reduce prevalence of AGD (by histological diagnosis) for 21 days after bathing. However, the efficacy of freshwater bathing is brought into question by a study in which amoebae levels returned to pre-bath numbers within 10 days of bathing (Clark et al., 2003).

The addition of chemicals to baths (chloramine-T, chlorine dioxide, and hydrogen peroxide) has also been investigated to increase the efficacy of bathing. Chloramine-T (CL-T), a widely

used chemotherapeutic and chemoprophylactic treatment for gill diseases in freshwater aquaculture (Thorburn and Moccia, 1993), has been added to freshwater and seawater baths and was found to increase the efficacy of bathing and reduce amoebae survival (Powell and Clark, 2003; Powell and Clark, 2004; Harris et al., 2005). Chlorine dioxide has also shown promise in reducing amoebae survival further than just freshwater, but higher concentrations are needed to significantly reduce amoebae survival compared to CL-T (25 mg L^{-1} compared to 10 mg L^{-1}) (Powell and Clark, 2004). Hydrogen peroxide was tested in freshwater (Powell and Clark, 2004) and seawater (Adams et al., 2012) and both were effective in ameliorating clinical signs of AGD in infected fish. However, hydrogen peroxide also needed a higher concentration (100 mg L^{-1}) and was found to be more toxic to Atlantic salmon than either chlorine dioxide or CL-T as evidenced by higher rates of mortality during the baths (Powell and Clark, 2004). Chemical additives remain a potentially useful avenue to reduce the cost, labour, and site limitations for salmon farmers as well as reducing stress on the fish due to handling.

Freshwater bathing is stressful to the fish and interrupts feeding, results in losses of growth, and can cause mortalities (Kube et al., 2012). The interval between baths is typically 35 to 40 days and a cohort of fish could need 8 to 13 baths during the 15 to 18 month marine grow-out period, making it a costly treatment method (Kube et al., 2012). Therefore, a selective breeding program was initiated in 2004 in Tasmania with the objective to breed for AGD resistant salmon, consequently extending the number of days between baths and decreasing the number of baths required during the marine phase of production (Elliott and Kube, 2009; Kube et al., 2012). Aside from disease resistance, traits for selection in the breeding program include growth (time to harvest), reducing occurrence of early maturation, and maintaining flesh quality (Elliott and Kube, 2009). Thermal tolerance is not a trait explicitly targeted for selection in the breeding program. The selective breeding program in Tasmania is predicted to increase the freshwater bathing interval by 3% every year (Kube et al., 2012).

Atlantic salmon farms in Tasmania are also increasingly producing more all-female triploid cohorts to provide a market supply year-round and to avoid early maturation (Nowak, 2012). The innate sterility of triploids allows fish to reach market size without diverting energy to maturation (Benfey, 2001), and the faster growth rates can conceivably result in less baths throughout the production cycle. However, while the use of triploids provides advantages to the industry, they have been reported to be more sensitive to AGD on farms

(Nowak, 2001). Indeed, an experimental infection found that mortality of triploids was greater and occurred earlier than their diploid counterparts (Powell et al., 2008). The reason behind the elevated mortality is unclear, however, as the percentage of gill filaments affected with AGD lesions of the triploids and diploids was similar throughout the experiment until day 28 when triploids exhibited less than that of the diploids (Powell et al., 2008).

Thermal dependence of infections

Warmer temperatures have been identified as one of the primary factors influencing the severity and duration of AGD outbreaks (Rodger and McArdle, 1996; Dyková et al., 1998; Clark and Nowak, 1999; Munday et al., 2001; Nowak, 2001). Other influencing factors are thought to be predisposing nodules or plaques, immune status, and stocking densities (Nowak and Munday, 1994; Findlay and Munday, 1998; Clark and Nowak, 1999; Findlay et al., 2000; Zilberg and Munday, 2000; Nowak, 2001) as well as low water exchange rates and poor husbandry practices (e.g. fouled nets) (Langdon, 1990). Clinical AGD has been documented in Atlantic salmon in temperatures ranging 15 to 20°C in Tasmania (Munday et al., 1990) and from 12 to 21°C in Ireland (Rodger and McArdle, 1996). The lower limit for AGD has been reported at 7-11°C, but mortality levels decrease to low levels (Steinum et., 2008). Amoebae have also been observed on Atlantic salmon gills in Tasmania in the winter months, but signs of clinical AGD were absent (no lesions) (Munday et al., 1990; Howard and Carson, 1993). Therefore, while the amoebae are capable of attachment at lower temperatures, lesions do not occur until warmer water temperatures are experienced, suggesting that the functional surface area of the gills may not be compromised at lower temperatures. In any event, knowledge of the interaction between temperature and AGD is almost exclusively based on farm observations, while investigations under controlled conditions remain scant.

Physiological effects of AGD

Amoebic gill disease was originally thought to cause mortality through respiratory failure (Powell et al., 2008). Despite the common symptoms of lethargy and respiratory distress in AGD-infected fish (Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996), early studies do not support respiratory failure as the physiological mechanism underlying AGD-related mortality in salmonids (Powell et al., 2000; Fisk et al., 2002; Leef et al., 2005b; Leef et al., 2007a). Although impaired gas exchange and respiratory acidosis have been observed

in AGD-infected Atlantic salmon through significantly lowered arterial oxygen partial pressure (P_{O2}), elevated carbon dioxide partial pressure (P_{CO2}) and lowered pH (Powell et al., 2000), there have only been minor differences in oxygen uptake reported between AGD-infected and naïve fish (Table **Error! Reference source not found.**) (Powell et al., 2000; Fisk et al., 2002; Leef et al., 2005b), suggesting that AGD-infected fish can defend respiration through physiological mechanisms (Powell, 2006). Booth (1978) reported only 58% of secondary lamellae of the rainbow trout gill were perfused with blood at rest. Therefore, AGD-infected fish, at least when resting, have substantial scope for recruitment of lamellae (Booth, 1979) or redistribution of blood flow to unperfused lamellae (Booth, 1979; Farrell et al., 1980) to preserve enough functional gill surface area to retain respiration. No significant differences in ventilation frequency have been observed in AGD-affected fish compared with control fish under normoxia (Powell et al., 2000; Fisk et al., 2002).

Notably, early studies of AGD and metabolism involved short-term respirometry with measurements taken periodically (typically 1 min intervals for 15 to 20 min) so may have lacked the robustness to see any differences (Powell et al., 2000; Fisk et al., 2002). Refined respirometry techniques, including continuous $\dot{M}O_2$ measurements over longer time periods, revealed an increase in standard and routine $\dot{M}O_2$ with progression of *Paramoeba* spp. infections in Atlantic salmon acclimated to ~15.5˚C (Leef et al., 2007c). In rainbow trout, an experimental reduction in functional gill surface area was directly related to a decrease in $\dot{M}O_{2max}$ (Duthie and Hughes, 1987; Schurmann and Steffensen, 1997), but earlier observations of AGD-infected Atlantic salmon show no such effect on $\dot{M}O_{2max}$ (Powell et al., 2005; Leef et al., 2007c) despite lesions decreasing surface area and hyperplasia presumably increasing the diffusion distance across the gill epithelium. A more recent study, however, has demonstrated a decrease in $\dot{M}O_{2max}$ in AGD infected fish compared to the controls using a U_{crit} protocol (Hvas et al., 2017a). With $\dot{M}O_{2max}$ remaining constant or decreasing and standard $\dot{M}O_2$ increasing with infection level, aerobic scope may become compromised. While Atlantic salmon reared on a farm may not have to utilise their full aerobic scope to undertake activities that their wild counterparts have to (e.g. upriver migrations or foraging), aerobic scope is still a relevant metric to measure for aquaculture-reared salmon. Farmed salmon may not have to forage, but they still have to out-compete each other for food or digest large meals which subsequently raises $\dot{M}O_{2rest}$ and decreases aerobic scope available for other activities. Being out-competed for food could potentially explain the lethargy seen in AGD-infected fish as mentioned above. Furthermore, it has been suggested

that heavily infected fish with AGD have limited abilities to deal with stressors (e.g. routine farm handling such as bathing, net cleaning, cage towing, as well as environmental factors such as abnormally high summer temperatures, low oxygen availability and changes in salinity) (Leef et al., 2007c) which could partially be explained by a lower aerobic scope.

Complications with cardiovascular function have also been implicated in causing AGD-related mortality. The heart is considered the powerhouse of the cardiovascular system (Yousaf et al., 2013), and a strong correlation has been established between morphology (e.g. ventricle mass) and function (e.g. cardiac output) of the organ (Graham and Farrell, 1992; Agnisola and Tota, 1994; Franklin and Axelsson, 1994; Sanchez-Quintana et al., 1995; Tota and Gattuso, 1996). Fish with a history of heavy AGD have been reported to exhibit altered morphometrics of the heart, whereby the ratios of ventricle axis length and width as well as axis length and height were significantly higher, and there was an overall thickening of the muscularis compactum (Powell et al., 2002b). While there is capacity for great morphological plasticity of the heart within a species (Poppe et al., 2003), any deviation from the pyramidal (triangular) shape, which is important for optimal cardiac functioning (Poppe et al., 2002), could predispose individuals to cardiac failure during periods of stress, such as AGD (Powell et al., 2008).

Despite the above observations, few studies have taken a controlled approach to investigate the effects of stressors (e.g. hypoxia, temperature) on AGD-infected Atlantic salmon in comparison with uninfected counterparts. Following a hypoxic challenge down to 50% oxygen saturation, severely AGD-infected fish (gill scores 2 to 4) had 21.4% survival compared with 88.9% survival of lightly infected fish (gill scores 0 to 1) (Fisk et al., 2002). However, there was a significant decrease in $\dot{M}O_2$ in AGD-infected fish under hypoxia compared with normoxia, so the authors suggested AGD-infected fish may have some scope for metabolic compensation (Fisk et al., 2002). Therefore, respiratory compromise remains to be proven as the cause of mortality but more likely creates other complications leading to death. However, no studies to date have examined the effect of AGD on the critical oxygen tension (P_{crit}; a measure of hypoxia tolerance), the O_2 concentration at which fish switch from oxy-regulators to oxy-conformers, the latter calling upon anaerobic metabolism for survival (Beckenbach, 1975). As with elevated temperatures, when faced with hypoxia, fish must increase the functional surface area of the gills to maintain adequate O_2 uptake. However, if the functional gill surface area is compromised, such as via lesions from AGD, then the P_{crit} may increase and cause the infected fish to switch to anaerobic metabolism at

a higher O_2 concentration. Furthermore, investigations into respiratory effects of AGD have been conducted at a single acclimation temperature (typically summer averages ~15 to 17°C; see Table **Error! Reference source not found.**), providing little insight into future effects of global warming. In addition, investigating physiology, such as respiratory capacity, under steady-state conditions does little to shed light on potential acute complications during the heatwaves projected under climate change models (Kirtman et al., 2013). Critical thermal maxima (CT_{max}) tests are common laboratory tests conducted to investigate the effects of acute temperature changes. While the rate of temperature increase during CT_{max} tests may exceed that experienced in the wild, there is evidence to indicate that CT_{max} estimates of thermal tolerance can provide insight into the performance of fishes under slower heating rates (see review by Terblanche et al., 2011). Therefore, investigating the effects of acute as well as chronic thermal tolerance of AGD-affected fish will help build understanding of climate change effects on aquaculture stocks suffering from AGD.

Table 1.2: Resting and maximum metabolic rates ($\dot{M}O_{2rest}$ and $\dot{M}O_{2max}$, respectively) of AGD-infected and control Atlantic salmon in the literature. (*) specifies if there was a significant difference between AGD and control values within that study.

Treatment Group	Weight (g)	Temperature (˚C)	$\dot{M}O_{2rest}$ (mg O_2 kg^{-1} h^{-1})		$\dot{M}O_{2max}$ (mg O_2 kg^{-1} h^{-1})	Reference
			Normoxia	Hypoxia		
AGD	1100 ± 460	17.0 ± 1.0	150.0 ± 1.0	130 ± 1.0		Fisk et al (2002)
Control			140.0 ± 1.0	100 ± 1.0		
AGD	911.7 ± 81.3	17.0 ± 1.0	139.8 ± 19			Powell et al (2000)
Control			160.0 ± 19			
AGD	123.1 ± 8.54	15.5 ± 0.5	174.1*		334.85	Leef et al (2007b)
Control			135.3		325.72	
AGD	118.1 ± 7.0	15.0	136.3*		319.13	Powell et al (2005)
Control			109.5		320.02	

Given that the current assessment and treatment of salmon for AGD involve handling stress and associated exercise, it is of interest to understand how AGD might influence the capacity of fish to recover from anaerobic exercise. Excess post-exercise oxygen consumption (EPOC) (Gaesser and Brooks, 1984; Gleeson and Hancock, 2002; Fu et al., 2009) is related to the ability to regain physiological homeostasis and replenish O_2 stores in blood and muscle tissues after anaerobic exercise (Børsheim and Bahr, 2003). Recovery time after exercise is also of ecological importance for wild populations because it may determine the ability of repeated activities crucial for survival and fitness (Milligan, 1996; Lee et al., 2003a; Lee et al., 2003b; Hanna et al., 2008; Fu et al., 2009). In the context of AGD, infected fish may have a reduced speed of recovery (prolonged EPOC) from exercise because of a lesion-induced decrease in gill oxygen uptake capacity, although the reported confusion regarding the influence of AGD on $\dot{M}O_{2max}$ points to a requirement for further research (Powell et al., 2005; Leef et al., 2007b; Hvas et al., 2017a).

Scope of Thesis

Aims and objectives

The primary aim of this thesis is to form a comprehensive understanding of the respiratory capacity and thermal tolerance of aquaculture-reared Atlantic salmon when challenged with AGD infection at acclimation temperatures relevant to climate change. In particular, studies in this thesis investigate how chronic and acute temperature regimes effect resting and maximal oxygen uptake rates and aerobic scope in AGD-infected individuals. Furthermore, this thesis is the first to quantify thermal tolerance of AGD-infected individuals and takes a first step towards understanding the impacts of AGD on hypoxia tolerance and recovery capabilities following anaerobic exercise. Finally, the thesis explores the metabolic function and thermal tolerance of diploid and triploid salmon with an aim to identify differences between ploidies that may underlie differential tolerance to environmental stressors.

Structure

This thesis encompasses three experimental chapters (Chapters 2 to 4) that are intended for peer-review publication. A version of Chapter 2 is accepted for publication at Journal of Experimental Biology. While all chapters have been written as independent publications, efforts have been taken to reduce repetition of introductory material and methods where possible.

Chapter 2 investigates the respiratory capacity and thermal tolerance of diploid and triploid Atlantic salmon parr during the freshwater phase of the lifecycle following acclimation to 10, 14, and 18˚C. Parameters measured include growth, $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, aerobic scope, and CT_{max}.

Chapter 3 examines the thermal tolerance of naïve and AGD-infected diploid Atlantic salmon through a CT_{max} protocol. In addition, subsets of fish are sampled throughout the temperature ramping protocol to understand the physiological responses of the two groups to the increase in temperature.

Chapter 4 quantifies the respiratory capacity of naïve and AGD-infected diploid Atlantic salmon at 15 and 19˚C. Specifically, aerobic capacity is determined through measurements of $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, and aerobic scope while hypoxia tolerance and anaerobic capacity are investigated by measuring P_{crit} and EPOC.

Chapter 5 is a general discussion that synthesises the new knowledge gained from the previous three chapters and places the findings in the context of prior knowledge. In particular, the chapter focuses on the key themes introduced in Chapter 1: metabolism, AGD, and environmental tolerance.

Chapter 2: Negligible differences in metabolism and thermal tolerance between diploid and triploid Atlantic salmon (*Salmo salar* L.)

The research within this chapter has been published as:

Bowden, A.J., Andrewartha, S.J., Elliott, N.G., Frappell, P.B., Clark, T.D. (2018). Negligible differences in metabolism and thermal tolerance between diploid and triploid Atlantic salmon (*Salmo salar* L.). Journal of Experimental Biology, jeb-166975.

Abstract

The mechanisms that underlie thermal tolerance in aquatic ectotherms remain unresolved. Triploid fish have been reported to exhibit lower thermal tolerance than diploids, offering a potential model organism to better understand the physiological drivers of thermal tolerance. Here, triploid and diploid juvenile Atlantic salmon (*Salmo salar* Linnaeus) were compared in freshwater to investigate the proposed link between aerobic capacity and thermal tolerance. Measurements were specific growth rates (SGR) and resting (aerobic) metabolic rates ($\dot{M}O_{2rest}$) in freshwater at 3, 7 and 9 weeks of acclimation to either 10, 14 or 18°C. Additionally, maximum metabolic rates ($\dot{M}O_{2max}$) were measured at 3 and 7 weeks of acclimation, and critical thermal maxima (CT_{max}) were measured at 9 weeks. Mass, SGR, and $\dot{M}O_{2rest}$ differed between ploidies across all temperatures at the beginning of the acclimation period, but all three metrics converged between ploidies by week 7. Aerobic scope ($\dot{M}O_{2max} - \dot{M}O_{2rest}$) remained consistent across ploidies, acclimation temperatures, and time. At 9 weeks, CT_{max} was independent of ploidy, but correlated positively with acclimation temperature despite the similar aerobic scope between acclimation groups. My findings suggest that acute thermal tolerance is not modulated by aerobic scope, and the altered genome of triploid Atlantic salmon does not translate to reduced thermal tolerance of juvenile fish in freshwater.

Introduction

Triploid fish have been proposed as useful experimental models to understand the mechanisms underlying environmental tolerance because their altered genome may influence tolerance levels under challenging conditions (Maxime, 2008). Indeed, triploid brown trout (*Salmo trutta*, Linnaeus) (adults in seawater) exhibited higher mortality rates than diploids when exposed to a high temperature challenge (18°C) for 3 weeks, and triploid mortalities reached 50% after 12 weeks at this temperature (Altimiras et al., 2002). Moreover, triploid rainbow trout (*Oncorhynchus mykiss*, Walbaum) (juveniles in seawater) suffered immediate mortalities when exposed to 21°C, and a total mortality of 69% was recorded after 3 weeks compared with 39% in diploid conspecifics (Ojolick et al., 1995).

Such observations raise the possibility of using triploids to elucidate the physiological mechanisms underlying temperature tolerance in fish. Triploid fish are typically produced by subjecting eggs to one or more pressure shocks within the first hour or two following fertilisation, resulting in the retention rather than extrusion of a polar body that gives rise to a third chromosome (Teskeredžić et al., 1993). Triploids compensate for this extra genetic material by having larger but fewer cells than their diploid counterparts, resulting in the two ploidies achieving similar sizes as adults (Swarup, 1959; Small and Benfey, 1987). Growth rates of triploids, however, have been inconsistent across studies and have been the same, greater than, or less than their diploid counterparts (Galbreath et al., 1994; McGeachy et al., 1995; McCarthy et al., 1996).

Furthermore, triploids are thought to have similar haematocrit (packed erythrocyte volume) as diploids but lower haemoglobin concentration, subsequently reducing blood oxygen carrying capacity (Benfey and Sutterlin, 1984a; Benfey et al., 1984; Graham et al., 1985). Blood oxygen transport of triploids may be further reduced due to the smaller surface area to volume ratio of the enlarged erythrocytes, which could inhibit oxygen diffusion dynamics (Sadler et al., 2000). In light of the proposed link between oxygen transport capacity and thermal performance, a logical extension is that inferior oxygen transport capacity in triploids drives their reportedly lower thermal tolerance (Pörtner and Knust, 2007). Having said that, the role of oxygen transport capacity in governing thermal tolerance is debated and requires additional investigation using different model organisms (Clark et al., 2013; Brijs et al., 2015; Ern et al., 2016).

Despite the apparent reduction in oxygen carrying capacity of triploid blood, contrasting results exist regarding their aerobic metabolic rates at both high and low acclimation temperatures. Higher routine metabolism has been reported in triploid Atlantic salmon (*Salmo salar*, Linnaeus) at an acclimation temperature of 12°C, but lower routine metabolism at a higher temperature (18°C) was considered to be indicative of lower thermal tolerance in triploids (Atkins and Benfey, 2008). In contrast, no differences in routine metabolic rate were found between diploid and triploid Atlantic salmon at 15°C, nor between diploid and triploid rainbow trout at 19°C (Benfey and Sutterlin, 1984b; Oliva-Teles and Kaushik, 1990). However, caution should be taken when comparing studies involving triploids. The method of triploid production can matter (e.g. temperature versus pressure shock) in terms of survival of eggs or larvae, rearing conditions, and husbandry (held together with diploids or separate) (see review by Maxime 2008). While maximum metabolic rate ($\dot{M}O_{2max}$) has received less attention, a study on Atlantic salmon reported no differences in $\dot{M}O_{2max}$ between ploidies at 15°C (Lijalad and Powell, 2009). Scant data and disparate findings point to a requirement for more comprehensive investigations to determine whether aerobic capacity may be causally linked with acute and/or chronic thermal tolerance across ploidies (Benfey et al., 1997; Galbreath et al., 2006).

Here, acute (representative of heatwaves) and chronic (representative of increased summer average temperatures) thermal tolerance of diploid and triploid juvenile Atlantic salmon was investigated with an aim to clarify some of the conflicting findings reported previously. Specifically, my objectives were first to compare the growth performance, resting metabolic rate ($\dot{M}O_{2rest}$), $\dot{M}O_{2max}$ and aerobic scope at three acclimation temperatures (10, 14, and 18°C), and then to quantify routine metabolic rate during assessments of critical thermal maxima (CT_{max}) in the different acclimation groups. The hypothesis was tested that triploids exhibit lower chronic and acute thermal tolerance than diploids, and that these differences are at least partly explained by lower aerobic capacity of triploids.

Materials and Methods

Animal husbandry

Diploid and triploid Atlantic salmon (n=35 and n=40, respectively) were sourced from the Salmon Enterprises of Tasmania Pty Ltd (SALTAS) freshwater hatchery in Wayatinah, Tasmania (water temperature ~9°C) and transported to Hobart, Tasmania. All experiments were conducted at the Aquaculture Physiology laboratory at CSIRO under

the animal ethics permit A0013794. Both groups were from the 2015 commercial spawning and had been incubated at ~8°C. The fish were separated by ploidy into two x 200 L tanks that were supplied by a freshwater recirculating system held at 10°C. After 7 days of recovery from transport, the fish were individually weighed (mean ± s.e.m.; diploids: 46.63 ± 2.31 g, triploids: 68.75 ± 2.08 g) and their fork lengths measured (diploids: 163.41 ± 2.95 mm, triploids: 188.63 ± 2.13 mm). Additionally, the fish were tagged intraperitoneally with a passive integrated transponder (PIT) to track individual performance and tagged with coloured elastomer in the adipose fin to visually discern ploidy (yellow for diploids and green for triploids) before being returned to their respective tanks for three days to recover from tagging. Thereafter, mixed groups of diploids and triploids (n=5 to 8 per ploidy) were assigned to six 68 L tanks (n=11 to 13 per tank) with recirculating freshwater and maintained at 10°C for three weeks.

Water temperature was subsequently raised to 14°C (to represent current-day summer sea temperatures of 14 to 15°C in southeast Tasmania) in two tanks and to 18°C (forecast of regional sea temperatures under a business-as-usual emissions scenario) in another two tanks (2°C d^{-1}) using 600 W titanium heaters with a digital Nema thermostat (Aquasonic, Wauchope, NSW, Australia). The remaining two tanks were maintained at 10°C to represent current-day winter temperatures. For each temperature, the heaters were placed in a common sump supplying the two tanks and the water maintained at ± 0.5°C of the desired temperature. Water was changed periodically to maintain water quality (ammonia: <0.7 mg L^{-1}; nitrite: <0.2 mg L^{-1}; nitrate: <20 mg L^{-1}). The fish were fed to satiation daily and were allowed to acclimate for three weeks to their respective temperatures prior to the experiments commencing. Dissolved oxygen was maintained above 85% air saturation and the lighting regime was kept at 10 h light and 14 h dark. During the ~4 weeks following tagging, there were 2 mortalities in the 10°C acclimation group (2 diploids), 2 in the 14°C acclimation group (1 diploid, 1 triploid) and 1 in the 18°C acclimation group (1 triploid). These mortalities were attributed to transport/tagging effects rather than to temperature, but these mortalities were not specifically investigated as this was not the main focus of the study. Furthermore, mortality was low enough that any statistical investigations would lack power.

Respirometry

Oxygen consumption rates of individual fish were measured in six intermittently-closed

respirometers (2.8 L) following previously-described methods (Clark et al., 2013). The respirometers were submerged in a 160 L temperature-controlled water bath that was maintained at >90% air saturation with compressed air. The temperature of the water bath was adjusted as necessary to match the acclimation temperature of the fish involved in the respirometry trial. Each respirometer was intermittently flushed every 15 min by a pump at 0.5 L min^{-1} to replenish the oxygen levels. Water was continuously mixed by a submersible pump (0.1 L min^{-1}) within a closed recirculation loop. Dissolved oxygen concentration was measured in each respirometer by fibre optic oxygen probes (FireSting O_2, Pyroscience, Aachen, Germany) sealed in the recirculation loop and recorded at 5 s intervals using an eight-channel PowerLab/8sp and LabChart 7 Pro software (ADInstruments Pty Ltd, Bella Vista, New South Wales, Australia).

Resting and maximum oxygen consumption rates were measured at three and seven weeks after the acclimation temperatures had been established. Fish from an acclimation temperature treatment were fasted for 24 hours and then dip-netted out of their holding tanks during mid-morning, anaesthetised with 0.02 mL L^{-1} Aqui-S (50% active isoeugenol) and their mass, length, and PIT tag number recorded. The fish were then placed in individual respirometers at their respective acclimation temperature and resting metabolic rate ($\dot{M}O_{2rest}$) was determined from oxygen consumption measurements (see below) during the 17 to 20 hour overnight recovery. The following morning, the fish were removed from the chambers and individually exercised in a 40 L round swim tank. Water temperature in the swim tank was maintained using an Aquasonic heater and Nema thermostat, and dissolved oxygen levels were maintained above 95% air saturation through aeration. A chase protocol was used to elevate oxygen consumption to the maximum level (Clark et al., 2013; Norin and Clark, 2016; Killen et al., 2017). Briefly, each fish was individually chased by hand for 2 min before being immediately placed back in the respirometer to measure the maximum oxygen consumption rate as a proxy for maximum metabolic rate ($\dot{M}O_{2max}$). All fish became exhausted by 2 min of vigorous chasing in a preliminary set of experiments (n=6). Exhaustion was recorded when the fish no longer swam away when being chased and tapped on the tail. Oxygen consumption measurements continued for 30 min to ensure the maximum rate of decline in oxygen concentration was captured. The fish were returned to their respective holding tanks after the respirometry protocol. Notably, a pump failure impacted one of the 14°C acclimation tanks between week 3 and week 7,

essentially halving the sample size at this acclimation temperature and resulting in n=6 diploids and n=8 triploids being measured at 14°C thereafter.

Critical thermal maxima

At nine weeks of acclimation, fish were placed in respirometers, as described above, to measure $\dot{M}O_{2rest}$, but a critical thermal maximum (CT_{max}) protocol rather than an exercise protocol was conducted the following morning. The temperature of the water bath containing the respirometers was increased stepwise by 2°C every 75 min (ramped increase over 15 min and held stable for 60 min to ensure stable oxygen measurements) and routine metabolic rate was measured over two 15-min periods during each 60 min interval once temperature had plateaued (similar to Brijs et al. (2015)). The protocol was ceased when the fish displayed loss of equilibrium (LOE), which was defined as the inability to right themselves after 10 s. The time and temperature at LOE were recorded and each fish was immediately euthanised via anaesthetic overdose (Aqui-S, 50% active isoeugenol). Blood was sampled from the caudal vasculature in a 1 ml heparinised syringe using a 27G needle, transferred to a 2 ml Eppendorf tube, and immediately placed on ice until processing (< 1 h).

Dissections and ploidy verification

Following LOE and blood sampling, the fish were dissected and the ventricle, liver, and spleen masses were recorded (Ohaus Scout Pro Portable Electronic Balance, Parsippany, NJ, USA). A sample (2 µL) of blood was smeared on a glass slide, dried, and stained in Diff Quik (Sigma Aldrich, Castle Hill, NSW, Australia) for ploidy verification. The blood smears were examined using light microscopy (Leitz Wetzler, Germany) and photographed using a Leica DFC310 FX microscope camera connected to a PC with Leica Application Suite Version 4.0.0 software (Leica Microsystems Limited, Switzerland). The photographs were processed using ImageJ 1.48v (Wayne Rasband, National Institutes of Health, USA). The nucleus major axis was measured for at least 50 randomly chosen erythrocytes per individual and triploids and diploids were statistically separated through cluster analysis on the mean nuclear length (Benfey et al., 1984).

Data analyses

Specific growth rate (SGR) was calculated using Eq. 2.1:

(2.1) $$SGR = [(\ln(M_f) - \ln(M_i)) \times t^{-1}] \times 100$$

where M_f and M_i are the final and initial masses, respectively, of an individual and t is the elapsed time between mass measurements in days.

Oxygen consumption rates (mg O_2 kg^{-1} h^{-1}) were calculated using slopes derived from linear regressions between oxygen concentration and time during each sealed event in the chamber and accounting for the volume of the respirometer as in Eq. 2.2:

$$\dot{M}O_2 = \frac{\frac{\Delta O_2}{\Delta t}(P_B - P_V)\, x\, \beta_{O_2}\, x\, Vol\, x\, 0.2093}{M_b} \quad (2.2)$$

where ΔO_2 is the change in oxygen concentration within the respirometer over the change in time in hours (Δt), P_B is the barometric pressure in kPa, P_V is the water vapour pressure (kPa, Antoine equation), β_{O2} is the calculated oxygen capacitance of freshwater at the acclimation temperature (mg L^{-1} kPa^{-1}; Dejours 1981), Vol is the volume of the respirometer minus that of the fish (assuming 1 kg wet mass = 1 L) in L, 0.2093 is the fractional concentration of oxygen in well-aerated water, and M_b is body mass (kg). Note that most of the statistical analyses were conducted using oxygen consumption rate in mg O_2 h^{-1} and body mass was included as a covariate (details below).

Resting metabolic rate ($\dot{M}O_{2rest}$) was determined as the mean of the lowest 10% of oxygen consumption values throughout the measuring period (17 to 20 hours), excluding outliers (values ± 2 s.d. from the mean (Norin et al., 2014)). Maximum metabolic rate ($\dot{M}O_{2max}$) was calculated from a 3 min slope immediately after the exhaustive chase protocol, which was always found to be the highest. Absolute aerobic scope was calculated by subtracting $\dot{M}O_{2rest}$ from $\dot{M}O_{2max}$, while factorial aerobic scope was calculated by dividing $\dot{M}O_{2max}$ by $\dot{M}O_{2rest}$.

Critical thermal maximum (CT_{max}) was calculated by modifying the critical swimming speed equation from Brett (1964) into Eq. 2.3:

$$CT_{max} = T_f + \left(\frac{t_f}{t_i} \times T_i\right) \quad (2.3)$$

where T_f is the highest temperature the fish endured for the full time period, t_f is the time the fish lasted at its final temperature step, t_i is the prescribed time for each temperature (i.e. 60 min), and T_i is the incremental temperature increase (i.e. 2°C).

Temperature coefficients (Q_{10}) were calculated to quantify the influence of acclimation temperature on the metabolism of diploids and triploids using mean $\dot{M}O_{2rest}$ values in Eq.

2.4:

$$(2.4) \quad Q_{10}=\left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2-T_1}}$$

where R_2 and R_1 are the mean $\dot{M}O_{2rest}$ values that correspond to two acclimation temperatures (T_2 and T_1). Q_{10} values were calculated between 10 and 18°C for each ploidy at each acclimation time point (3, 7 and 9 weeks).

All data are presented as mean ± 95% confidence intervals in figures and text. Organ weights were analysed using ANCOVAs (see below) and presented as body mass adjusted means from the ANCOVA outputs.

Statistical analyses

Mass and SGR data were analysed using a series of two-way ANOVAs and ANCOVAs, respectively. The two factors (ploidy and acclimation temperature) were analysed within each time point and alpha for significance was set at 0.05 / 3 tests = 0.017. ANCOVAs for SGR used the initial mass as the covariate. Differences in metabolic rates were analysed using general linear mixed models, testing metabolic rate (mg O_2 h^{-1}) against sampling time point, ploidy, and acclimation temperature with mass as a covariate and accounting for repeated measures on an individual. Results are reported from the repeated measures analyses using F tests (type III Wald F tests with Kenward-Roger degrees of freedom approximation). If applicable, post hoc tests for pairwise comparisons using Bonferroni corrections were utilised to investigate the differences between ploidies within time points and the differences across time points within ploidy. CT_{max} data were analysed using a two-way ANOVA because there was no time point variable. Measurements of routine metabolic rate (mg O_2 kg^{-1} h^{-1}) during the CT_{max} protocol were averaged for each 60-min interval and then analysed with a linear mixed effects model to test the effect of temperature (covariate) increase on metabolic rate between ploidies (factor) with individual as a random factor to control for repeated measures. Organ weights were analysed using ANCOVAs testing the absolute values against ploidy and acclimation temperature with mass as a covariate. Significance was accepted at $p<0.05$ unless otherwise indicated (for multiple tests) and Bonferroni corrected post hoc tests were conducted on covariate-adjusted means where applicable. All analyses were conducted using R Studio (Version 1.0.136) with R packages *car* (Fox and Weisberg, 2011), *nlme* (Pinheiro et al., 2016), and *lsmeans* (Lenth, 2016).

Results

Ploidy verification

The erythrocyte nuclei measurements confirmed that the ploidy of all fish was correctly classified (Fig. 2.1). In this study, the major axis of the nucleus in triploids was 1.48 times longer than that of diploids, which compares favourably with a value of 1.26 times longer reported in Benfey et al. (1984).

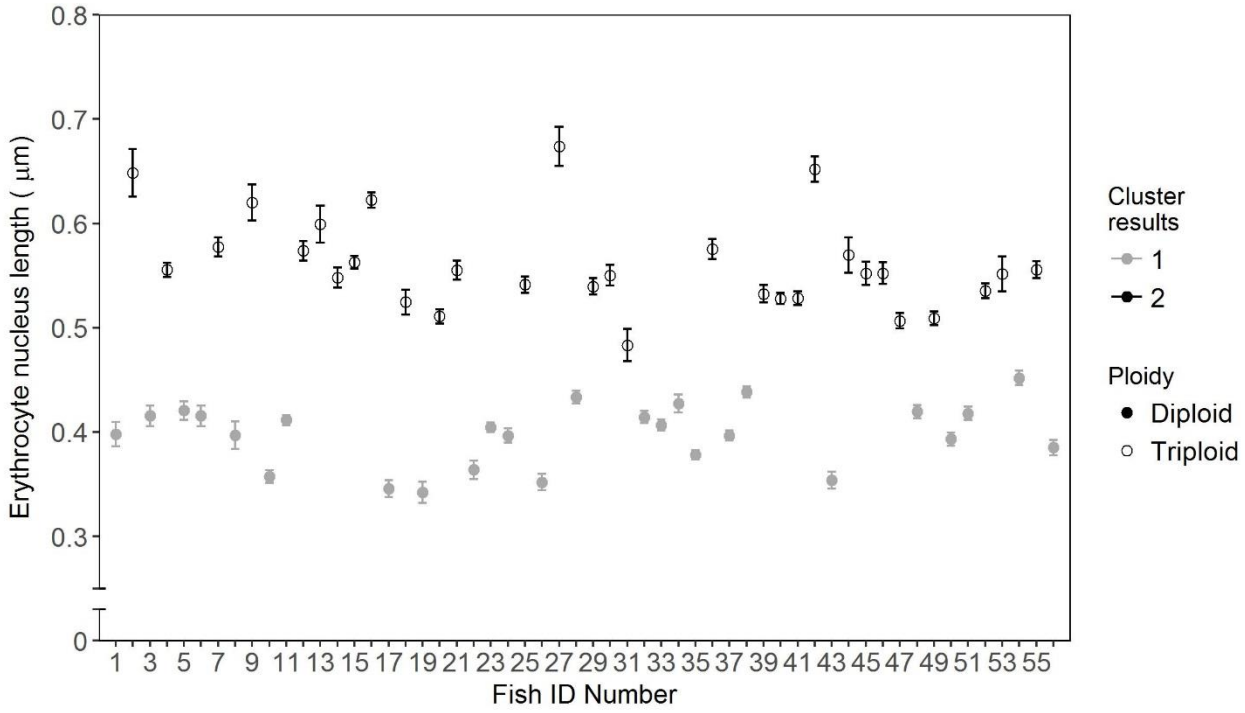

Figure 2.1: Mean erythrocyte nucleus length for each individual. Colours represent the k means clustering results. Closed grey circles are assumed diploids while open black circles are assumed triploids from the beginning of the experiment. Points are mean ± S.E.M. of all the nuclei measured for one individual.

Survival, mass and growth

There were no natural mortalities in either ploidy at the 10 or 14°C acclimation temperatures once the experiment commenced. There were two triploid mortalities at 18°C between weeks 7 and 9, although the low mortality rate makes it difficult to attribute these deaths to a lower chronic thermal tolerance of triploids.

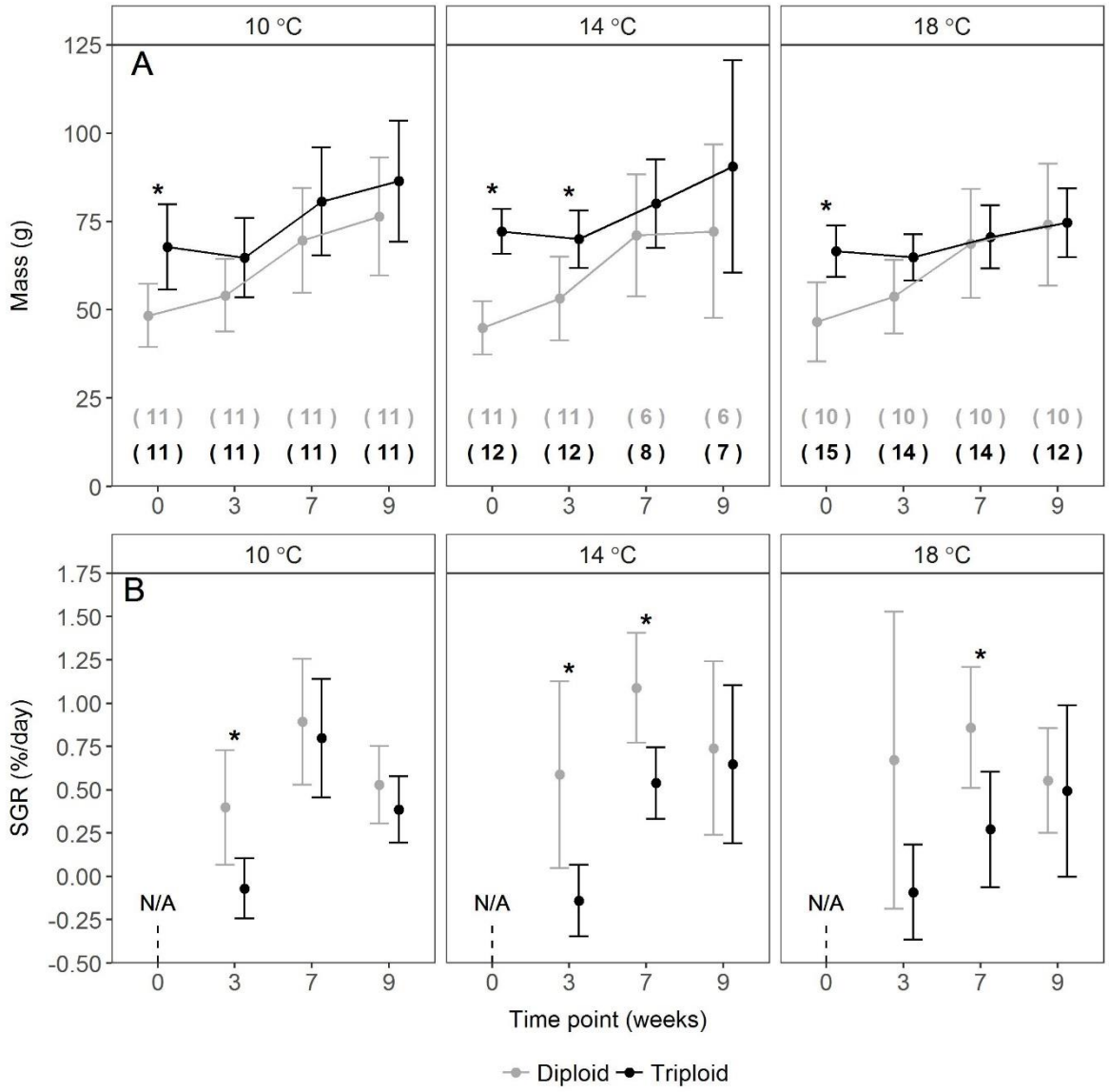

Figure 2.2: (A) Mass and (B) specific growth rate (SGR) for diploid (grey) and triploid (black) Atlantic salmon during 9 weeks of temperature acclimation to 10, 14, and 18°C. Samples sizes are in parentheses in (A). All values are means ± 95% confidence intervals and positioned side by side to reduce overlap for clarity. (*) demarcates significance between ploidies based on ANOVAs (mass) and ANCOVAs (SGR) with alpha reduction for multiple testing (see Methods). Note that in (B), values represent SGR between time points (e.g. from 0 to 3 weeks) and therefore could not be calculated for week 0 (N/A)

Triploid mass was ~1.5 times greater than diploids at week 0 across all acclimation temperatures (Fig. **2.2**A, 10°C: $F_{1,18}$=9.01, p=0.008; 14°C: $F_{1,21}$=37.58, p<0.001; 18°C: $F_{1,23}$=11.91, p=0.002). Triploids and diploids acclimated to 10 and 18°C were similar sizes at week 3 and remained similar at subsequent time points. On the other hand, triploid fish acclimated to 14°C continued to be larger than diploids at week 3 ($F_{1,21}$=6.93, p=0.016) but the masses of the ploidies converged by week 7. The temporal dynamics in fish mass across the ploidies and acclimation temperatures were consistent with SGR of the different groups (Fig. **2.2**B).

Relative ventricle, liver, and spleen masses were similar between diploids and triploids across all acclimation temperatures. Not surprisingly, gonad mass was significantly greater in diploids than in the inherently sterile triploids at all acclimation temperatures (Table 2.1, 10°C: $F_{1,17}$=54.32, p<0.001; 14°C: $F_{1,6}$=17.68, p=0.006; 18°C: $F_{1,13}$=56.77, p<0.001).

Metabolic rates

As expected, there was a general increase in $\dot{M}O_{2rest}$ with acclimation temperature from ~55 to ~125 mg kg^{-1} h^{-1} in both ploidies (Q_{10} between 10 and 18°C (week 3, week 7, and week 9, respectively) = 1.75, 1.75 and 2.31 for diploids; 1.87, 1.73 and 1.99 for triploids). Counter to my expectation that $\dot{M}O_{2rest}$ would decrease as fish progressively acclimated to the higher temperatures, there were some significant increases in $\dot{M}O_{2rest}$ between week 3 and week 7 (~75 to ~115 mg kg^{-1} h^{-1}) in both ploidies at 14°C (diploids: $F_{2,79}$=10.96, p=0.004; triploids: $F_{2,79}$=34.87, p<0.001) and in triploids at 18°C (~96 to ~120 mg kg^{-1} h^{-1}: $F_{2,79}$=21.61, p<0.001; Fig. **2.3**A).

On average, $\dot{M}O_{2rest}$ was 15% higher in diploids compared with triploids across all acclimation temperatures during the week 3 measurements (Fig. **2.3**A, 10°C: $F_{1,62}$=10.20, p=0.002; 14°C: $F_{1,62}$=7.84, p=0.007; 18°C: $F_{1,62}$=12.72, p=0.001). Diploids also had a higher $\dot{M}O_{2rest}$ at 10°C during the week 7 measurements (10°C: $F_{1,62}$=6.39, p=0.014), but $\dot{M}O_{2rest}$ was similar between ploidies at 14 and 18°C. There were no differences in $\dot{M}O_{2rest}$ between diploids and triploids during the week 9 measurements.

Table 2.1: Body mass adjusted means (from ANCOVA) for the organ masses of diploid and triploid Atlantic salmon acclimated to three different temperatures. Values are presented as a percentage of body mass and are mean ± 95% confidence intervals. (*) denotes significant differences between ploidies within a temperature.

Measurement	Acclimation temperature (°C)					
	10		14		18	
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid
Ventricle (%)	0.077 ± 0.007	0.076 ± 0.007	0.092 ± 0.012	0.091 ± 0.012	0.069 ± 0.006	0.070 ± 0.005
Liver (%)	0.858 ± 0.110	0.943 ± 0.110	1.054 ± 0.221	1.053 ± 0.204	0.636 ± 0.101	0.788 ± 0.093
Spleen (%)	0.084 ± 0.018	0.101 ± 0.019	0.184 ± 0.068	0.093 ± 0.049	0.073 ± 0.018	0.069 ± 0.017
Gonad (%)	0.193 ± 0.023*	0.075 ± 0.023	0.217 ± 0.042*	0.093 ± 0.059	0.239 ± 0.034*	0.046 ± 0.044

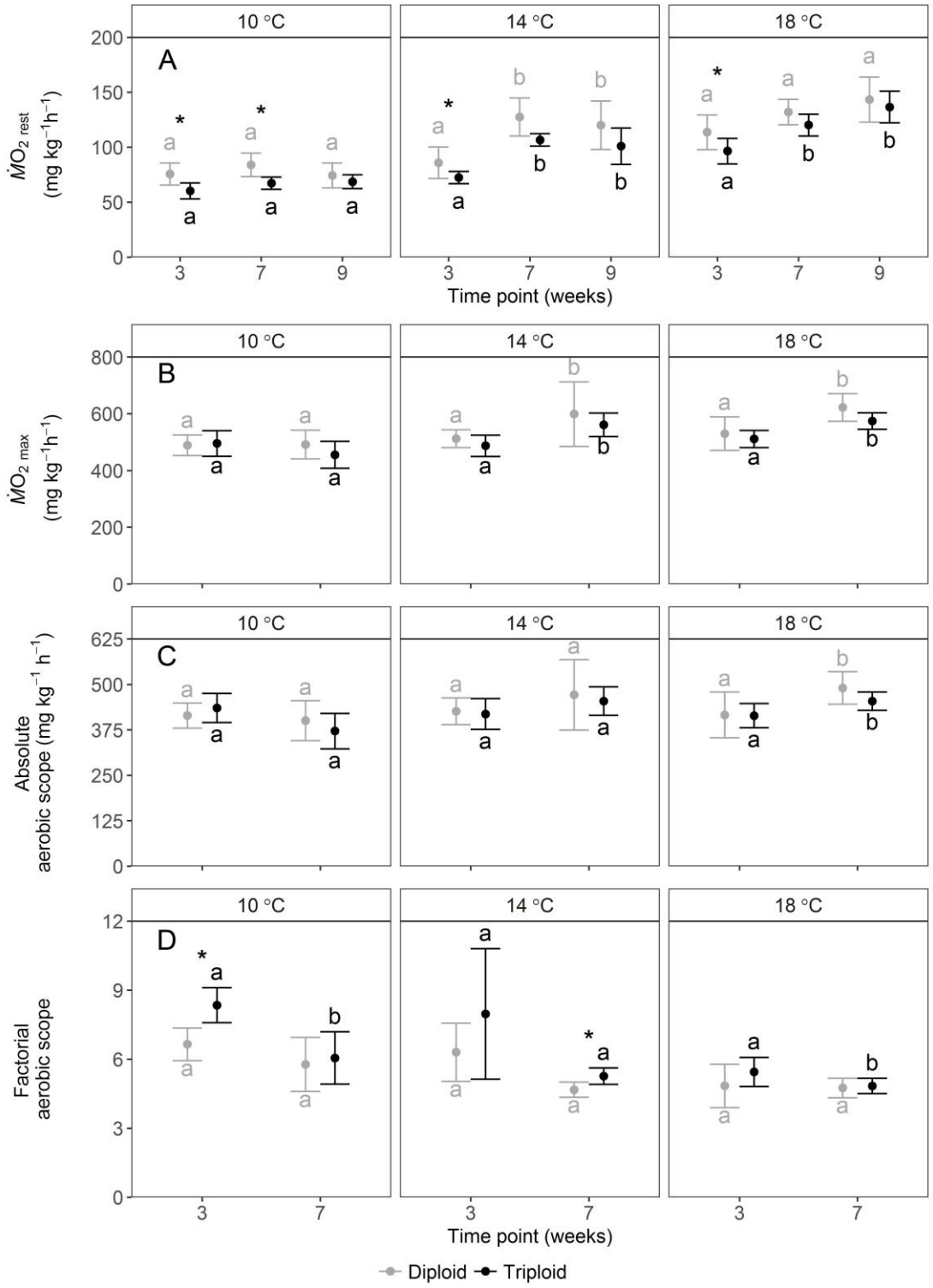

Figure 2.3: (A) Minimum oxygen consumption ($\dot{M}O_{2rest}$), (B) maximum oxygen consumption ($\dot{M}O_{2max}$), (C) absolute aerobic scope, and (D) factorial aerobic scope for diploid (grey) and triploid (black) Atlantic salmon measured during acclimation to 10, 14, and 18°C. Values are mean ± 95% confidence intervals. Significance between ploidies is denoted by (*) and differences between measuring time points (weeks) within a ploidy are signified by different lower-case letters (Bonferroni p-value adjustments for pairwise comparisons). See Fig. **2.2**A for sample sizes.

Absolute aerobic scope and $\dot{M}O_{2max}$ were generally stable across temperatures (~400 mg kg^{-1} h^{-1} and ~500 mg kg^{-1} h^{-1}, respectively) for both ploidies (Figs. **2.3**B, C), while there was a tendency for a ~25% decline in factorial aerobic scope across the range of acclimation temperatures (Fig. **2.3**D). Within ploidies, there were small but significant increases in $\dot{M}O_{2max}$ between weeks 3 and 7 at 14 and 18°C, and an increase in absolute aerobic scope between weeks 3 and 7 at 18°C (Figs. **2.3**B, C; $\dot{M}O_{2max}$: 14°C: diploids: $F_{1,40}$=4.63, p=0.038; triploids: $F_{1,40}$=7.85, p=0.008; 18°C: diploids: $F_{1,40}$=17.04, p<0.001; triploids: $F_{1,40}$=10.83, p=0.002; aerobic scope at 18°C: diploids: $F_{1,40}$=15.85, p<0.01; triploids: $F_{1,40}$=4.46, p=0.041).

Between ploidies, $\dot{M}O_{2max}$ and absolute aerobic scope were similar at all acclimation temperatures within both measuring time points (weeks 3 and 7) (Figs. **2.3**B, C). Factorial aerobic scope, however, was higher in the triploids compared with diploids within the 10°C acclimation group at week 3 (Fig. **2.3**D, $F_{1,18}$=11.29, p=0.047) and within the 14°C acclimation group at week 7 ($F_{1,11}$=6.62, p=0.026). There was some evidence of factorial aerobic scope decreasing between weeks 3 and 7 in triploids at 10°C (Fig. **2.3**D, 10°C: $F_{1,13.4}$=11.45, p=0.001).

Critical thermal maxima

CT_{max} did not differ between ploidies (Fig. **2.4**A, $F_{1,51}$=1.76, p=0.190), but increased with acclimation temperature from ~26 to ~29°C ($F_{2,51}$=66.27, p<0.001). Thus, there was generally a ~0.4°C improvement in CT_{max} for every 1°C increase in acclimation temperature. Within diploids, CT_{max} was similar between fish acclimated to 10 and 14°C, but higher for fish acclimated to 18°C ($p_{10\text{-}18°C}$<0.001, $p_{14\text{-}18°C}$<0.001). Thermal effects were more consistent for triploids, whereby CT_{max} increased significantly between the 10 and 14°C acclimation groups and between the 14 and 18°C acclimation groups ($p_{10\text{-}14°C}$=0.016, $p_{14\text{-}18°C}$=0.001).

Consistent with the findings for $\dot{M}O_{2rest}$ described above, diploids had a higher routine metabolic rate than triploids during the CT_{max} protocol when commencing at the acclimation temperatures of 14 and 18°C. In contrast, there were no differences in routine metabolic rate between the ploidy groups acclimated to 10°C (Fig. **2.4**B, 10°C: $F_{1,20}$=0.002, p=0.969; 14°C: $F_{1,11}$=5.115, p=0.045; 18°C: $F_{1,20}$=6.676, p=0.018).

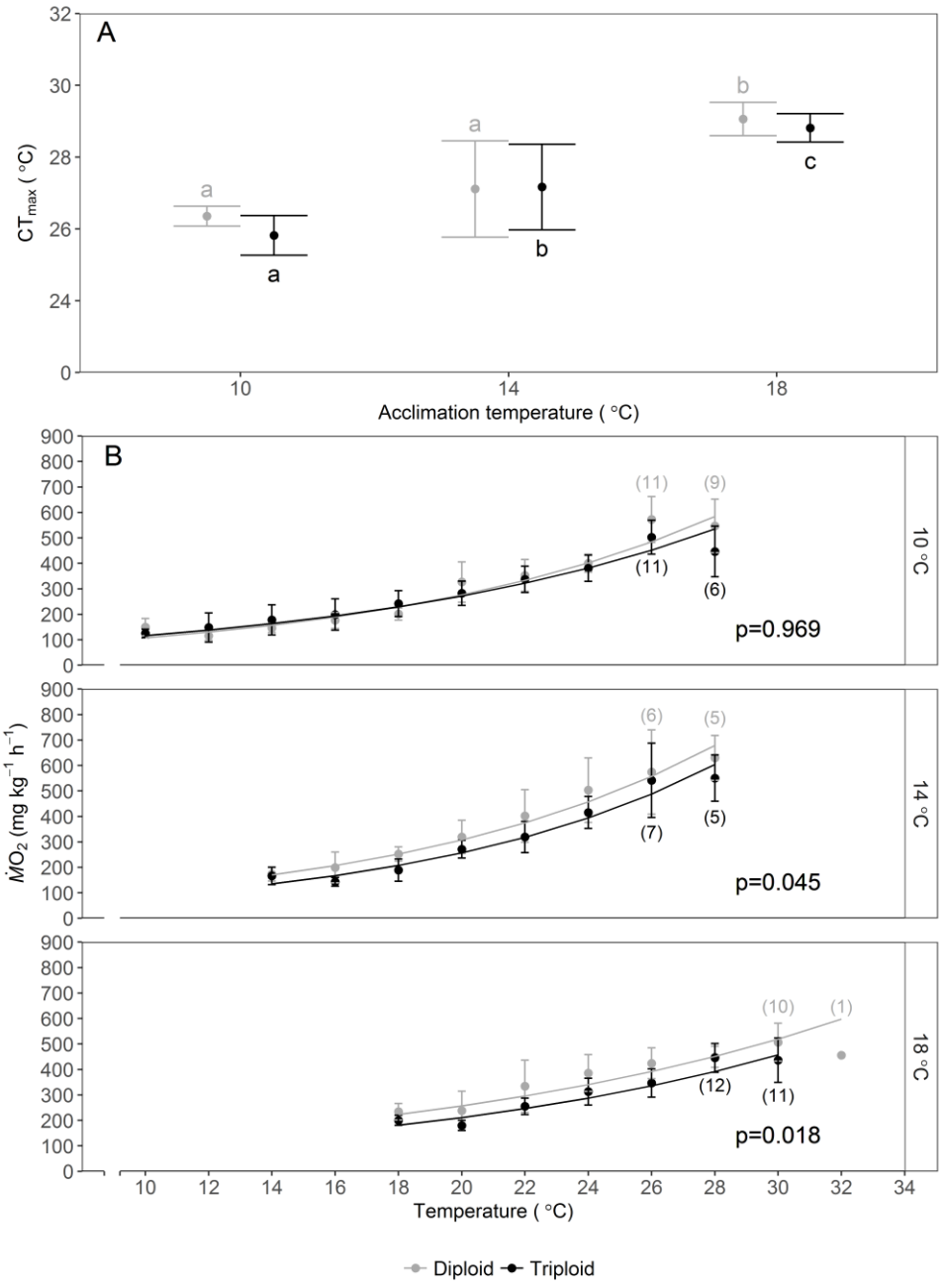

Figure 2.4: (A) CT_{max} temperatures for diploid (grey) and triploid (black) Atlantic salmon across acclimation temperatures and (B) oxygen consumption rate during the CT_{max} protocol. (A) Values are mean ± 95% confidence intervals. Lower case letters show differences within a ploidy across acclimation temperatures. (B) Values are mean ± 95% confidence intervals fitted with exponential regressions with the equations: diploids at 10°C: $y = 42.385(0.088)*e^{0.094(0.004)x}$ (R^2=0.84); triploids at 10°C: $y = 50.135(0.099)*e^{0.085(0.004)x}$ (R^2=0.81); diploids at 14°C: $y = 42.571(0.114)*e^{0.099(0.005)x}$ (R^2=0.92); triploids at 14°C: $y = 30.291(0.133)*e^{0.107(0.006)x}$ (R^2=0.86); diploids at 18°C: $y = 63.089(0.186)*e^{0.070(0.007)x}$ (R^2=0.60); triploids at 18°C: $y = 45.139(0.157)*e^{0.077(0.006)x}$ (R^2=0.68). P-values represent significance between the two regressions. Numbers in parentheses indicate when sample sizes decreased.

Discussion

In contrast with my initial hypothesis, triploids performed similarly to their diploid counterparts when held chronically at three acclimation temperatures and during the CT_{max} challenge. My finding of similar thermal performance between ploidies is consistent with some previously published studies on Atlantic salmon, although there is some evidence for species differences (McGeachy et al., 1995; Ojolick et al., 1995; O'Flynn, 1997; Altimiras et al., 2002). Ploidy differences in the present study were observed for mass, SGR, and $\dot{M}O_{2rest}$, but not for $\dot{M}O_{2max}$ and aerobic scope (Figs. **2.2**, **2.3**). Text below discusses how these parameters may interact to result in subtle but important differences between ploidies.

Growth and metabolism

As expected, $\dot{M}O_{2rest}$ increased with acclimation temperature in both ploidies, a trend that is well documented for fishes (Fry, 1971; Clarke and Johnston, 1999; Gillooly et al., 2001). Interestingly, there was a difference between the ploidies for $\dot{M}O_{2rest}$ within an acclimation temperature in the first periods of the study (weeks 3 and 7) but not at the end (week 9). This is similar to the temporal trends in mass and SGR (Figs. **2.2**, **2.3**A), indicating there may be some common mechanisms between the measured parameters. Indeed, food consumption, growth and $\dot{M}O_{2rest}$ are thought to be intrinsically linked (Metcalfe et al., 1995; Pedersen, 1997; Yamamoto et al., 1998; Van Leeuwen et al., 2012; Norin et al., 2016).

The lower $\dot{M}O_{2rest}$ of triploids indicates a lower maintenance cost per unit body mass, which should theoretically translate to higher growth rates for a given energy intake assuming equivalent assimilation efficiencies. However, diploids rather than triploids displayed a higher SGR in the earlier weeks of the experiment when the $\dot{M}O_{2rest}$ of triploids was lower. It is possible that these counter-intuitive findings are a consequence of fish size, as growth rates are known to be negatively related to body size in fish (Iwama, 1996a). Furthermore, the significantly larger gonads of the diploids compared to the triploids is unlikely to have contributed to the higher $\dot{M}O_{2rest}$ as the fish utilised in this study were still juveniles and far from being sexually mature. Indeed, when diploids caught up to triploids in mass by week 7, SGR and $\dot{M}O_{2rest}$ also converged, supporting the idea that it was fish size rather than ploidy that drove the differences in the earlier weeks of the experiment.

Nevertheless, it is also possible that behavioural differences between the ploidies played some role in the counter-intuitive findings. Specifically, diploid salmonids have been reported to out-perform their triploid counterparts when raised in mixed-ploidy populations, resulting in improved growth

of diploids (Carter et al., 1994; Galbreath et al., 1994). This potential behavioural difference between ploidies may explain why investigations into growth between triploids and their diploid conspecifics have been largely inconclusive, whereby triploid Atlantic salmon have been reported to grow faster, slower, and similarly to their diploid counterparts (Galbreath et al., 1994; McGeachy et al., 1995; McCarthy et al., 1996). That is, the disparate findings may be largely attributed to husbandry practices such as keeping the ploidies separate versus mixing them in a common tank (Galbreath et al., 1994; Maxime, 2008). For example, Atlantic salmon triploids grew at a faster rate and had a higher mass when kept in a separate tank than diploids, whereas diploids out-performed the triploids when the two groups were mixed (Galbreath et al., 1994). In instances where diploids out-performed triploids in mixed tanks, it has been attributed to a more aggressive nature of diploids (Galbreath et al., 1994). In this context, intraspecific competition and dominance status has been correlated with standard metabolic rate, such that individuals with an inherently higher standard metabolic rate (as with the diploids in this study) are stimulated to eat more food and thus be more competitive/aggressive (Metcalfe et al., 1995; Cutts et al., 1998; Norin et al., 2016). In any event, similar growth rates have also been observed between ploidies of Atlantic salmon and coho salmon (*Oncorhynchus kisutch*, Walbaum) when the ploidies are mixed in tanks, highlighting that further controlled experiments are required on this topic to tease apart the roles of ploidy, body size, $\dot{M}O_{2rest}$ and behaviour (Johnson et al., 1986; Carter et al., 1994).

Acute thermal tolerance and aerobic capacity

Ploidy did not influence the CT_{max} of Atlantic salmon in this study, despite triploids from the 14 and 18°C acclimated groups maintaining a slightly lower routine metabolic rate during the temperature ramp (Fig. **2.4**). The lower routine metabolic rate is consistent with the lower $\dot{M}O_{2rest}$ observed for triploids in the earlier weeks of acclimation (Fig. **2.3**A). Despite the common observations that triploids suffer higher mortality at elevated temperatures, few studies have found ploidy differences within CT_{max} protocols with a variety of heating rates (e.g. 2°C h^{-1}, 15°C h^{-1}, 2°C d^{-1}) and species (e.g. rainbow trout, brook char, brook trout) (Benfey et al., 1997; Galbreath et al., 2006; Maxime, 2008; Scott, 2012; Scott et al., 2015). However, the CT_{max} experiments conducted here and in previous studies used juvenile fish, and it is known that smaller individuals can be more thermally tolerant than their adult conspecifics (Clark et al., 2012; Messmer et al., 2016; Clark et al., 2017). Furthermore, adult salmonids face other energetically costly physiological processes such as sexual maturation and ion regulation in a hypertonic environment upon their movement from fresh to salt water during their later life stages (Bœuf and Payan, 2001). While the enlarged erythrocytes of triploids do not seem to impair oxygen transport capacity, they could hinder other critical functions

like ion regulation. Therefore, it is possible that experimental results from juvenile triploids in freshwater may not be directly applicable to adult triploids in marine environments. Future research would benefit from focussing on marine environments to help establish differences across the life cycle.

Another consideration is that this study investigated metabolism and CT_{max} exclusively under normoxic conditions, whereas salmon in wild or cultured environments may be subjected to periods of hypoxia. In this context, upper thermal limits have been proposed to be oxygen-limited when in normoxic environments (Pörtner and Knust, 2007). Nevertheless, CT_{max} of red drum (*Sciaenops ocellatus* Linnaeus) and marine lumpfish (*Cyclopterus lumpus* Linnaeus) was found to be independent of oxygen availability over a wide range of oxygen tensions (Ern et al., 2016). Indeed, CT_{max} was not affected by oxygen availability until close to (lumpfish) or below (red drum) the species-specific critical oxygen tensions (P_{crit}) despite significant decreases in aerobic scope (72 and 89% reductions, respectively) (Ern et al., 2016). Given that it would be rare for Atlantic salmon in natural or cultured environments to experience oxygen levels below their P_{crit} (~35% air saturation for the size used here; (Barnes et al., 2011)), my findings should be applicable across the range of oxygen levels Atlantic salmon typically experience throughout their lifecycle.

Interestingly, a larger relative ventricle mass has been correlated with higher temperature tolerance in fish (Ozolina et al., 2016). It has been suggested that a deterioration in cardiovascular performance may be causal to the upper thermal tolerance limits of fishes (Lannig et al., 2004; Clark et al., 2008a; Farrell, 2009). Indeed, enhancing oxygen availability to the heart of European perch has been shown to play a role in maintaining stroke volume at critically high temperatures. Nevertheless, a concurrent decline in heart rate was likely to reflect direct temperature effects rather than an oxygen limitation (Ekström et al., 2016). Since ventricle mass is positively correlated with stroke volume (Graham and Farrell, 1989), and there were no differences in ventricle mass between ploidies in the present study (Table 2.1), it suggests that stroke volume was similar between triploids and diploids at the three acclimation temperatures. As such, there was no evidence that enhanced cardiovascular performance played any role in increasing CT_{max} with acclimation temperature (Fig. **2.4**). Having said that, there is some recent evidence that larger hearts can actually reflect poorer health and performance in fishes (Johansen et al., 2017). Furthermore, contrasting results for relative ventricle mass between ploidies have been reported, ranging from significantly heavier in Atlantic salmon triploids, to similar among ploidies for rainbow trout and Atlantic salmon (Fraser et al., 2015; Verhille et al., 2013; Fraser et al., 2013). The heart is a highly plastic organ, so the contrasting observations could be due to environmental conditions such

as rearing temperature, salinity, and natural versus controlled environmental challenges (Fraser et al., 2015).

Values of CT_{max} increased by approximately 0.4°C with every 1°C increase in acclimation temperature in both triploids and diploids, which is consistent with the changes in CT_{max} observed with acclimation temperature in previous studies of salmonids (Lutterschmidt and Hutchison, 1997; Currie et al., 1998). Nevertheless, aerobic scope remained stable across acclimation temperatures in both ploidies, suggesting that increased aerobic capacity was not responsible for the increase in CT_{max} of the fish acclimated to the higher temperatures (Fig. **2.3**C). This contrasts with the idea of oxygen- and capacity-limited thermal tolerance (OCLTT), which assumes that temperature-dependent performance and thermal tolerance are governed by aerobic scope (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). The lack of linkage between aerobic scope and CT_{max} adds to a growing database indicating that thermally-dependent fitness is primarily driven by factors other than oxygen supply capacity (Clark et al., 2013; Lefevre, 2016; Sandblom et al., 2016). This conclusion also concurs with a recent paper that found oxygen limitation is not the defining factor of thermal tolerance for post-smolt Atlantic salmon in seawater (Hvas et al., 2017b).

Conclusions and future directions

This study shows that the thermal tolerance of juvenile Atlantic salmon in freshwater is similar between diploids and triploids, and it does not appear to be influenced by aerobic capacity. It also shows that differential growth rates between ploidies can emerge when ploidies are mixed within the same tanks. Based on this and previous studies, future efforts to understand reported differences in thermal tolerance between ploidies should utilise large size ranges, freshwater vs. saltwater, and mixed-ploidy vs. single-ploidy tank arrangements. Moreover, investigations should examine different physiological attributes in concert with the oxygen transport cascade. For example, Sambraus et al. (2017) found lower ion (Cl^-, Na^+, and K^+) concentrations and higher glucose levels in blood plasma of seawater-acclimated triploid compared with diploid post-smolt Atlantic salmon at warm temperatures, suggesting lower physiological tolerance in triploids. Therefore, osmotic challenges (parr-smolt transformation, spawning migration of adults) could be investigated in conjunction with thermal stress to illuminate any role of ion regulation in determining differential ploidy survival at high temperatures. Such multi-stressor occurrences also exist during different phases of salmon aquaculture (e.g. freshwater influx in coastal aquaculture facilities), where triploids are increasingly favoured due to the advantages gained by their inherent sterility (Benfey, 1999; Benfey, 2001). The findings and suggestions highlighted here should pave

the way for future studies to further ascertain the extent to which triploids represent a useful model for elucidating the mechanisms underlying environmental tolerance in fish.

Chapter 3: Advanced stages of amoebic gill disease reduce the acute thermal tolerance of Atlantic salmon, *Salmo salar* L.

Abstract

Amoebic gill disease (AGD; caused by *Paramoeba perurans*) is the leading health issue facing Atlantic salmon (*Salmo salar* Linnaeus) aquaculture in the coastal regions of Tasmania, Australia. High mortality rates occur in the summer when the disease proliferates simultaneously with lower freshwater influx and higher temperatures in coastal systems. To better understand the reported link between AGD infection and temperature, the thermal tolerance of AGD-infected and non-infected (control) Atlantic salmon were tested using a critical thermal maxima (CT_{max}) protocol. Subsets of infected and control fish were sampled at four temperatures throughout the protocol: 17°C (before temperature ramping), 21°C, 25°C, and at CT_{max}. Blood samples were taken at each test temperature to determine haemoglobin, haematocrit, plasma cortisol and lactate levels. Individuals with high infection loads had a lower CT_{max} than those with low infection loads and larger controls (determined by breakpoint analysis as >222 g), but no differences were detected between heavily infected individuals and smaller controls (<222 g). There were also no differences in the measured blood parameters across treatment groups. Further research should clarify any performance impacts of initial exposure to *P. perurans* and early-stage infections, as well as the physiological mechanisms that cause AGD-associated mortality at elevated temperatures.

Introduction

Temperature has been termed an 'ecological master factor' because of its critical role in governing the performance and fitness of ectothermic animals (Brett, 1971; Deutsch et al., 2008). Thus, it is not surprising that climate warming and the increasing occurrence of summer heatwaves has significantly shifted the distribution of many ectothermic animal populations, especially those in aquatic environments (Perry et al., 2005; Sunday et al., 2012). Magnifying the effects of warming in aquatic environments is an increase in disease risk, which may be linked with a decrease in host immunity or an increase in virulence of pathogens and parasites (Karvonen et al., 2010). This 'arms race' between hosts and their pathogens/parasites can also be influenced through interactive effects between temperature and other environmental parameters such as dissolved oxygen and salinity (Snieszko, 1974; Harvell et al., 2002). Despite these increasing challenges, some species are not able to relocate to more favourable environments because they have exclusive associations with home sites (e.g. many coral reef fishes) or they are confined to artificial systems (e.g. aquaculture facilities). Therefore, understanding the impacts of temperature and disease on these species is important for forecasting how changing environments may impact species viability across natural and exotic distributions.

Atlantic salmon, *Salmo salar* L., is a dominant aquaculture species around the world. In Tasmania, Australia, a global warming 'hotspot' (Hobday and Pecl, 2014), the intensity and duration of heatwaves have been increasing throughout recent decades (Oliver et al., 2017). Indeed, the summer of 2015/2016 experienced the most extreme heatwave on record with 251 days above the historic average (Oliver et al., 2017). In this region, and to a lesser extent in some other countries, cultured Atlantic salmon face a significant health risk due to amoebic gill disease (AGD), a condition that seems to be proliferating as aquatic environments increasingly experience chronic and acute elevations in temperature (Munday et al., 1990; Rodger and McArdle, 1996; Clark and Nowak, 1999). While outbreaks of AGD have occasionally been reported at relatively cool temperatures (~10°C) in some parts of the world (Douglas-Helders et al., 2001), the disease is considered a summer problem in Tasmania where the impact is greatest. As stated in the General Introduction, the disease is caused by *Paramoeba perurans* (Young et al., 2007), causing slightly raised, white mucoid patches on the gills during outbreaks (Adams and Nowak, 2001). These lesions effectively compromise the gills by decreasing the functional surface area available for gas and ion exchange (Kent et al., 1988; Adams and Nowak, 2001; Adams and Nowak, 2003).

Temperatures around 15°C are thought to be optimal for *P. perurans*, and temperatures exceeding ~15°C have been reported to increase the prevalence of AGD and the infection rates of Atlantic

salmon (Douglas-Helders et al., 2001). To compound the challenge for salmon, increased water temperature causes a decrease in dissolved oxygen concentration while simultaneously increasing metabolic requirements (Fry, 1971; Wetzel, 2001). Thus, an increased prevalence of temperature spikes in association with reduced oxygen availability could amplify the challenge to infected salmon with compromised gills (Fisk et al., 2002). Heavily infected salmon, therefore, may be unable to cope with elevated temperatures compared to their control counterparts or lightly infected individuals, and they may be characterised by elevated blood stress indices associated with stress and anaerobic metabolism (e.g. cortisol, lactate, erythrocyte swelling). To date, however, no studies have quantified differences in the thermal tolerance and blood parameters of AGD-infected salmon in comparison with control conspecifics.

In order to assess the potential effects of temperature spikes on farmed Atlantic salmon, a critical thermal maximum (CT_{max}) test was utilised. A CT_{max} test provides insight into the thermal physiology of organisms negating the confounding factors of thermal acclimation and behavioural regulation of body temperature (Lutterschmidt and Hutchinson 1997) and has been found to correlate with thermal ranges experienced by fishes in their natural environments (Sunday et al., 2012). Here, a CT_{max} protocol was utilized to investigate how AGD impacts acute thermal tolerance and blood parameters using Atlantic salmon from aquaculture facilities in Tasmania, Australia. Specifically, a continuum of disease states was used to understand whether the CT_{max} is compromised above a certain infection level. It was hypothesized that control fish have the highest thermal tolerance and that acute thermal tolerance decreases linearly with increasing infection level in parallel with blood stress indices.

Methods

Animals, husbandry and infection

Fingerling Atlantic salmon (~5 g) were shipped by air from Tasmania to the CSIRO Bribie Island Research Centre, Queensland, Australia, in November 2014 and transferred to a 5000 L freshwater recirculation system tank. Water quality was monitored daily and recorded (ammonia <0.25 ppm, nitrite <0.25 ppm and DO 80-100% saturation). The salmon were fed a commercial salmon feed diet to satiation daily and grown to ~50-60 g. When the population was deemed ready for seawater acclimation, the tank was switched from freshwater recirculation to seawater flow-through at a rate of ~10 L min^{-1} to gradually increase salinity to 35 ppt overnight. The fish were then maintained at 15°C and grown to ~200 g in the 5000 L circular tank on a partial recirculation system with 20% seawater exchange per day. In November 2015, subsets of

fish to be infected were taken from the control holding tank and transferred to four completely flow-through 1000 L circular tanks with similar stocking densities (~11 kg m^{-3}). AGD infection was induced via cohabitation with previously infected fish, and the disease was allowed to progress for three weeks to achieve variation in disease states within the tanks (Findlay et al., 1995; Zilberg and Munday, 2000; Roberts and Powell, 2005; Leef et al., 2007b). Overall, 29% of fish held in the infection holding tanks died before the experiments commenced (fish were not weighed), while negligible mortality (<5%) occurred in the control stock tank. Mortalities from infection tanks were investigated and deemed a result of AGD infection as evidenced by substantial coverage of the gills by lesions as well as wet mounts from gill swabs that confirmed the presence of *P. perurans*. All tanks were maintained at 15°C and food was provided at ~0.5% body weight per day.

Experimental set-up

Control (n=15 to 30, per trial) and infected (n=39 to 50, per trial) fish were moved into two 300 L experimental tanks which were supplied in parallel by a submersible pump positioned in a single 800 L sump. Vigorous aeration was provided to the experimental tanks and the sump at all times and at no point did dissolved oxygen drop below 88% air saturation. Water pumped into the experimental tanks (68 L min^{-1} per tank) overflowed through a standpipe and returned to the sump. There were approximately twice as many infected fish in each of the three trials, so the control experimental tank was split in half by a mesh barrier to keep stocking density (fish biomass per volume of water) similar between the two groups. The experimental tanks received flow-through water (~10 L min^{-1}) during overnight acclimation (~12 hours) to prevent ammonia build-up (< 0.07 mg L^{-1}). The flow-through rate was progressively reduced throughout the CT_{max} trial the following day to achieve desired heating rates. Water temperature was controlled in the sump by four heaters (two 1000 W and two 600 W titanium heaters, Aquasonic). Temperature was recorded throughout the experiment in both experimental tanks and the sump using iButtons (Maxim Integrated, San Jose, CA, USA). The water temperature rose and stabilized at 16.5 to 16.9°C during the overnight acclimation period due to heat output from the pump and ambient air temperature.

Experimental protocol

In the morning of each trial, after at least 12 h of post-handling recovery, any mortalities that occurred overnight were removed from the experimental tanks and the entire gill basket assessed and scored for AGD using standard farm criteria (Taylor et al., 2009b) (Table 3.1).

Subsequently, 3 to 4 controls and 6 to 10 infected fish per trial (herein termed 17°C fish) were sampled from the experimental tanks, euthanised, gill scored, and samples collected (see below). The water temperature was thereafter increased at a rate of 2°C h^{-1} as suggested as a suitable heating rate for salmonid parr (Elliott and Elliott 1995). Further fish were sampled at 21 and 25°C (3 to 4 controls and 6 to 10 infected per trial at each temperature). The remaining fish were monitored until they reached their CT_{max}, which was determined as the temperature where loss of equilibrium (LOE) occurred and was maintained for 10 s. Once fish reached their CT_{max} they were removed from the tank, euthanised, gill scored, and samples were taken (see below). The critical thermal maxima (CT_{max}) trial was replicated on three consecutive days to achieve desired sample sizes of 64 fish from control and 132 fish from infected groups to ensure a range of gill scores were sampled (Table 3.2).

Table 3.1: Gill score criteria to determine AGD severity modified from Taylor et al (2009).

Gill score	Infection level	Gross appearance
0	Clear	Gills show no sign of infection and appear healthy
1	Very light	1 white spot or undefined necrotic streaking
2	Light	2 to 3 white spots or mucous patch
3	Moderate	Up to 20% of gill area covered by mucous patches
4	Advanced	Established lesions covering 20 to 50% of gill surface area
5	Heavy	Over 50% of gill area covered by mucous patches

Table 3.2: Total sample sizes of control and infected individuals during sampling protocol.

Treatment	Overnight mortalities	17°C	21°C	25°C	CT_{max}
Control	6	10	10	10	28
Infected	4	19	17	17	78

Blood samples

Blood was sampled from the caudal vasculature using a 22 G needle and a 4 mL lithium-heparinised vacutainer and immediately placed on ice until processing (<1 h). Haemoglobin concentration ([Hb]) was measured using the HemoCue^{TM} haemoglobin analyser (HemoCue 201^{+}, Ängelholm, Sweden). Since the HemoCue is designed to measure human haemoglobin concentrations and thus requires calibrating for fish blood (Clark et al., 2008b), equation 3.1 was taken from Andrewartha et al. (2016) to correct the values for salmon:

(3.1) $y = 0.820\,x - 5.831$

where *y* is the corrected haemoglobin value and *x* is the value measured with the HemoCue. Haematocrit (Hct) was measured using 16 µL of whole blood spun at 11,000 rpm for 1 min (SpinCrit Microhematocrit Centrifuge, USA). Subsequently, mean corpuscular haemoglobin concentration (MCHC) was calculated using equation 3.2:

(3.2) $MCHC = [Hb]/(Hct/100)$

The remaining blood from each sample was then pipetted into 2 mL Eppendorf tubes and centrifuged at 12,000 rpm for 5 min (Sigma 2-16P, Sigma Laboratories, Germany). The plasma was frozen at -20°C for the remainder of the experiment before being transferred to a -80°C freezer for long-term storage (5 months) and subsequent analysis of cortisol and lactate concentrations.

Plasma cortisol and lactate concentrations were determined as indicators of stress and anaerobic activity. Cortisol was measured using a commercial kit (Arbor Assays DetectX Cortisol Enzyme Immunoassay, BioScientific, Kirrawee, NSW, Australia). Samples were thawed on ice, diluted 1:300 with the supplied assay buffer, and then assayed as per the manufacturer's instructions using a 96-well plate reader (SpectraMax 190 Microplate Reader, Molecular Devices, Sunnyvale, California, USA). Lactate was determined using a 96-well plate assay. First, a standard curve was generated by adding 0, 2, 4, 6, and 8 µL of 5 mM lactate stock solution in triplicate followed by 200 µL of the reaction buffer (glycine buffer, H_2O, and NAD^{+}) to produce final concentrations of 0, 49.5, 98, 145.6, and 192.3 µM. Samples were diluted with the reaction buffer 1:30 and then 4 µL added in triplicate to the 96-well plate with a further 200 µL of reaction buffer. The plate was incubated at room temperature for 30 min. Afterwards, 10 µL of LDH was added to each well followed by a 30 min incubation at 37°C. Absorption was read at

340 nm on a 96-well plate reader (SpectraMax 190 Microplate Reader) and the standard curve utilized to determine final concentrations of plasma lactate.

Organ weights

All fish were measured for their mass (Scout Pro Digital Balance, Ohaus Australia) and fork length after sampling. All fish sampled prior to CT_{max} were dissected and their ventricle, liver, and spleen masses were recorded. Blood was squeezed from the ventricle before weighing. Only the first five and last five fish to reach CT_{max} each day were dissected due to the high number of fish to be processed at this time point. The second left gill arch of all sampled fish was excised and fixed in Davidson's seawater fixative for 24 h before being transferred to 70% ethanol for long term storage. Gill arches were subsequently examined under a dissecting microscope (Nikon TMS) to confirm the presence of AGD lesions.

Analysis

Condition factor (k) was calculated from body mass and length using equation 3.3:

(3.3) $$k = 100M_b/L^3$$

where M_b is body mass (g) and L is fork length (cm) (Fulton, 1904). Individuals with a condition factor less than 0.7 (10 out of 197 fish) were deemed unhealthy according to salmonid industry guidelines and thus they were omitted from subsequent analyses (Acharya, 2011).

Two AGD-exposed fish from the CT_{max} tests had not developed lesions, so they were classified as a gill score 0 and omitted from the CT_{max} statistical analysis due to insufficient sample size. Furthermore, CT_{max} was dependent on body mass within the control group but not in any of the AGD infection groups (determined using regression analyses of CT_{max} as a function of body mass), so control fish were split into two subgroups based upon mass with 222 g as the cut-off (as determined by breakpoint analysis; 'segmented' package in R). The influence of gill score on CT_{max} was tested using a one-way ANCOVA with mass as a covariate. Data for CT_{max} were Box-Cox transformed to satisfy the assumptions of normality and homogeneity of variance. Due to limited sample sizes at the three sample temperatures prior to CT_{max}, fish were pooled within each temperature into three groups of infection level, herein referring to the gill score groups, for the haematological and organ weight data: gill scores 0 and 1 were deemed as 'light', 2 and 3 as 'medium' and 4 and 5 as 'heavy' (see Table 3.1). Blood parameters were analysed using a series of two-way ANOVAs testing the parameter against infection level and sampling temperature. The effects of infection level and sampling temperature on organ weights were

analysed using two-way ANCOVAs with mass as a covariate. There was no effect of temperature on organ mass, so the data were pooled across temperatures to test the effect of infection level. Tukey HSD post hoc tests were used for all multiple pairwise comparisons where applicable. All analyses were performed using R Studio Version 00.99.879 using R packages nlme (Pinheiro et al., 2016), car (Fox and Weisberg, 2011), lsmeans (Lenth, 2016), and segmented (Muggeo, 2003).

Results

Survival and fish condition

There were significant differences between infection classifications for fish mass and condition factor, but not fork length (Table 3.3; mass: $F_{3,173}$=3.871, p=0.0103; condition factor: $F_{3,170}$=4.057, p=0.0081; fork length: $F_{3,170}$=2.524, p=0.0519). On average, all infection groups had higher condition factor than control fish, but only fish exhibiting medium infection levels (gill scores 2 or 3) were significantly heavier (p=0.006). The 20% difference in mass between the control and medium-infected fish was associated with an ~8% increase in condition factor.

Table 3.3: Sample sizes and morphological measures for Atlantic salmon of control, light, medium, and heavily infected individuals. Gill scores are in parentheses and values are presented as mean ± S.E.M. Letters demarcate significance within a parameter.

Infection level	n	Mass (g)	Fork length (mm)	Condition factor
Control (C)	55	193.23 ± 12.79^{a}	270.98 ± 4.46	0.909 ± 0.017^{a}
Light (0-1)	21	212.47 ± 14.28^{ab}	281 ± 5.18	0.93 ± 0.022^{ab}
Medium (2-3)	45	243.48 ± 13.68^{b}	287.71 ± 4.56	0.98 ± 0.016^{b}
Heavy (4-5)	61	218.31 ± 9.32^{ab}	281.27 ± 3.81	0.951 ± 0.012^{ab}

Furthermore, 3.8% of fish died in the infection tank (178.74 ± 27.08 g) and 7.7% of fish died in the control tank (111.12 ± 4.73 g) during the overnight acclimation period at 16 to 17°C across the three experimental trials (Fig. **3.1**). This higher mortality in control fish likely reflects the selection that occurred in the infection stock tanks prior to the experiments (29% mortality), whereby 'weaker' (often smaller) fish succumbed to the AGD infection and left 'stronger' (often larger) fish available for the CT_{max} trials, while the full range of weak-to-strong phenotypes remained alive in the control group. Evidence for this idea is presented in Fig. **3.1**, where small control fish are characterised by high levels of overnight mortality and lower CT_{max} compared with similar-sized heavily-infected fish. Indeed, breakpoint analyses on the control group and each of the AGD infection groups revealed that only the control group had a statistically significant breakpoint mass at which CT_{max} changed. Thus, the control group is divided into two subgroups ('small' and 'large') when discussing CT_{max} herein.

Thermal tolerance

Gill score significantly influenced the thermal tolerance (i.e. CT_{max}) of Atlantic salmon (Fig. **3.2**; $F_{6,86}$=7.703, p<0.001). Fish exhibiting gill score 5 lost equilibrium at a lower temperature (mean ± S.E.M., 26.8 ± 0.3°C) than larger control fish (28.24 ± 0.2°C, p=0.010) and those with lower gill scores of 1, 2, and 3 (28.6 ± 0.1°C, 28.6 ± 0.2°C, and 28.5 ± 0.2°C, respectively) (p=0.002, p=0.003, p=0.010, respectively). CT_{max} remained statistically similar between fish of gill scores 1 through 4. There was no significant difference in CT_{max} between smaller control fish (<222 g) and those at the extreme gill score of 5.

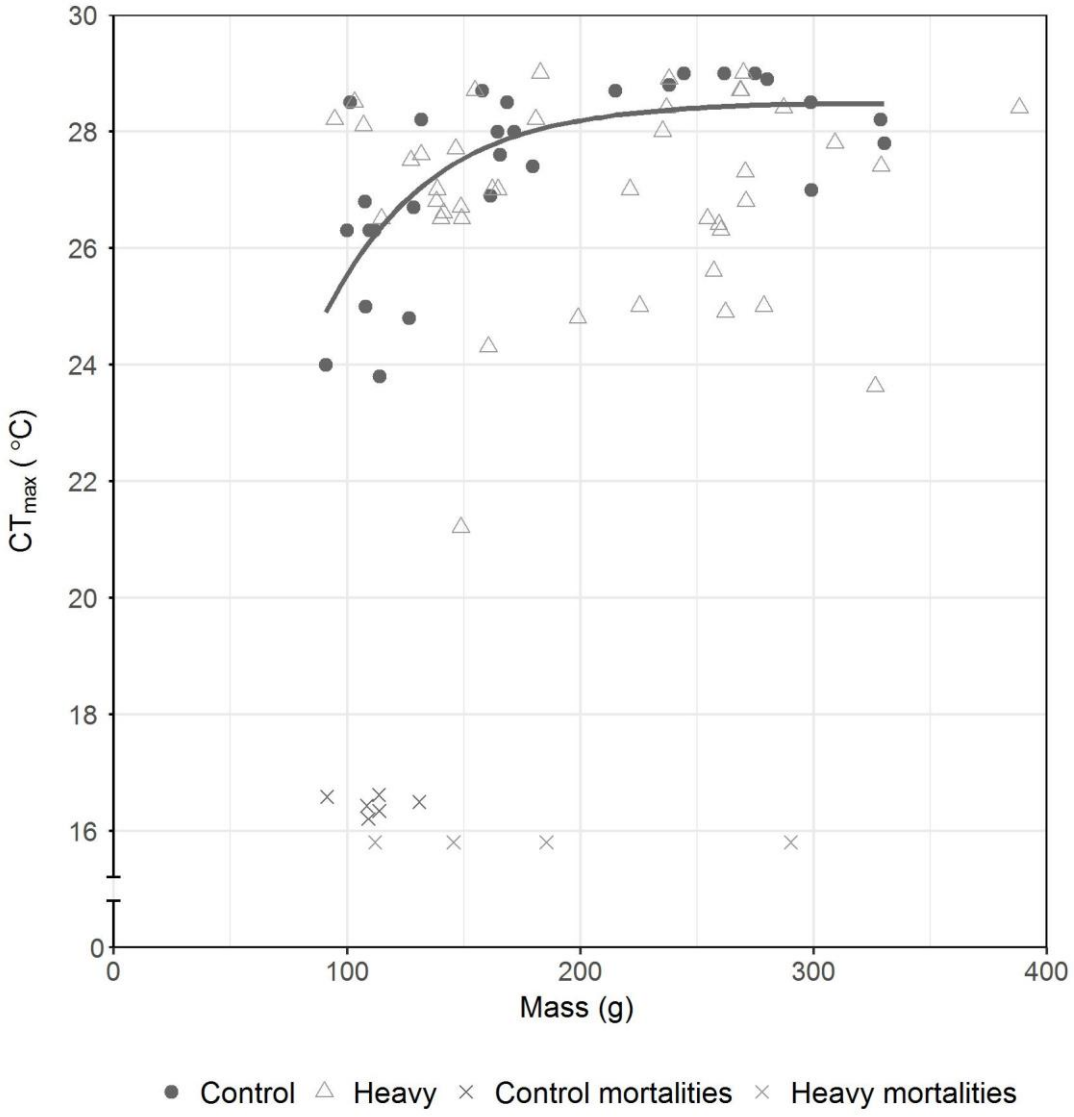

Figure 3.1: CT_{max} temperatures by mass for control (●) and heavily infected (△) Atlantic salmon. The regression line for control fish is represented by the equation: $y=28.49*(1- e^{-0.02x})$. There was no significant regression found for heavily infected fish or any of the other infection levels. Mortalities that occurred overnight in the experimental tanks during the recovery period at 16-17°C are represented by X. Mortality points are offset from each other on the vertical axis to prevent overlap (control indicated just above 16°C, infected just below 16°C).

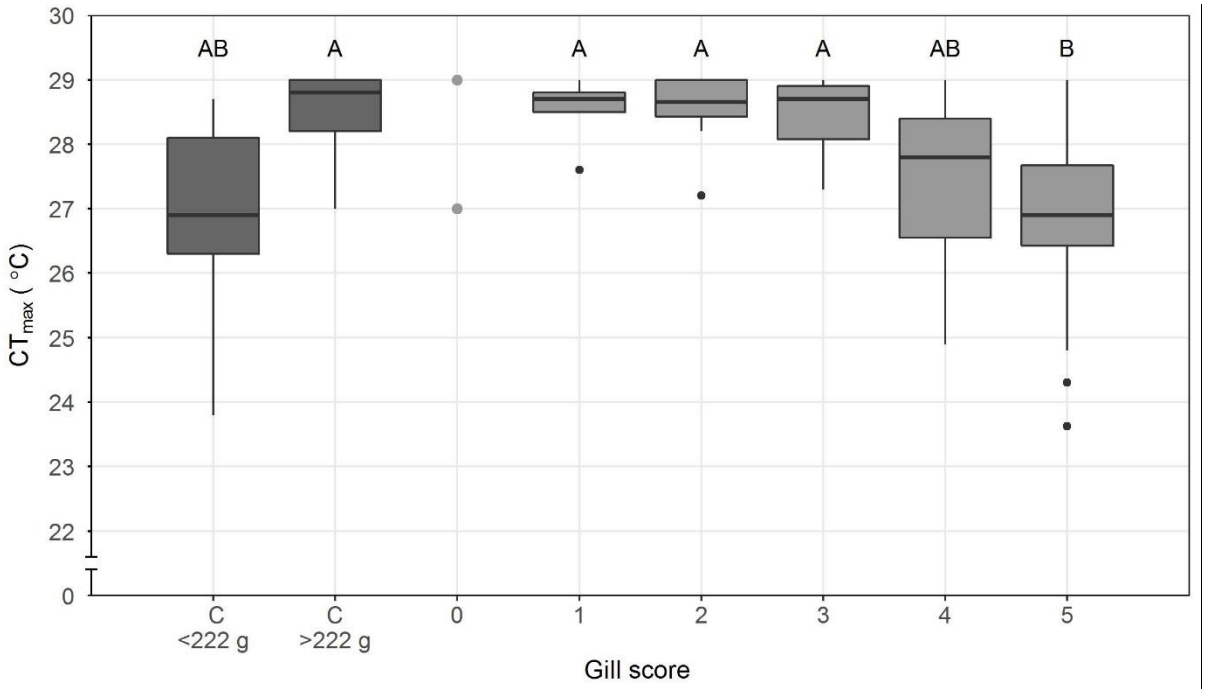

Figure 3.2: Box and whisker plot of CT_{max} of control (C; dark grey box) and AGD-infected Atlantic salmon (gill score 0 represented as points (n=2, not included in statistical analysis) and gill scores 1 to 5 represented as light grey boxes). Boxes represent the inter-quartile range (25^{th} to 75^{th} percentiles) and whiskers are the minimum and maximum values excluding outliers (filled circles). Letters demarcate similar significance based upon the statistical difference between the means of each group.

Haematological responses

Sampling temperature had an overall effect on haemoglobin (Hb), haematocrit (Hct), and MCHC (Fig. **3.3**A-C; Hb: $F_{3,166}$=4.861, p=0.003; Hct: $F_{3,166}$=18.252, p<0.001; MCHC: $F_{3,166}$=32.308, p<0.001). Lightly infected fish drove the temperature-related difference in [Hb] because concentrations were higher at their CT_{max} (132.2 ± 4.3 g L^{-1}) compared to those measured at 21°C (111.0 ± 4.8 g L^{-1}) (Fig. **3.3**A). Haematocrit increased in all treatment groups except in heavily-infected fish where it remained stable over the warming protocol. Control and medium-infected fish exhibited significant increases in Hct at 25°C which then remained stable to CT_{max} (Fig. **3.3**B). Patterns in MCHC were generally counter to the changes in Hct (Fig. **3.3**C), suggestive of erythrocyte swelling. MCHC in control fish was significantly lower at 25°C when compared to the control levels at 17°C, whereas MCHC in infected fish did not become significantly different from 17°C until they reached their CT_{max} (Fig. **3.3**C).

Plasma cortisol was highly variable and fluctuated in all treatment groups over the course of the CT_{max} protocol. There was no effect of infection level on cortisol levels, but there was an overall difference between sampling temperatures (Fig. **3.3**D; $F_{3,111}$=6.388, p<0.001). The difference was primarily driven by the medium infection group where fish at 21°C had significantly lower cortisol levels (93.2 ± 7.7 ng mL^{-1}) than fish at 25°C (249.3 ± 28.1 ng mL^{-1}) or at their CT_{max} (272.3 ± 38.1 ng mL^{-1}). Plasma lactate increased significantly with sampling temperature but there were no differences between infection levels (Fig. **3.3**E; $F_{3,147}$=13.701, p<0.001). All treatment groups except the medium infection group had significantly higher plasma lactate at their CT_{max} compared to the starting temperature of 17°C.

Organ masses

Relationships between organ masses as a function of body mass were best described by power functions (Fig. **3.4**). The absolute mass of the liver had an almost isometric relationship with body mass (slope (*b*)= 1.07 ± 0.086), while the relationship for ventricle mass was less than isometric (*b*= 0.758 ± 0.045) and the relationship for spleen mass was greater than isometric (*b*= 1.120 ± 0.079) (Figs. **3.4**D-F). Infection level did not have a significant effect on liver, ventricle, or spleen masses ($F_{3,68}$=2.269, p=0.088; $F_{3,69}$=2.067, p=0.113; $F_{3,62}$=1.142, p=0.339, respectively, including fish mass as a covariate), as illustrated by relative organ masses in Figs. **3.4**A-C.

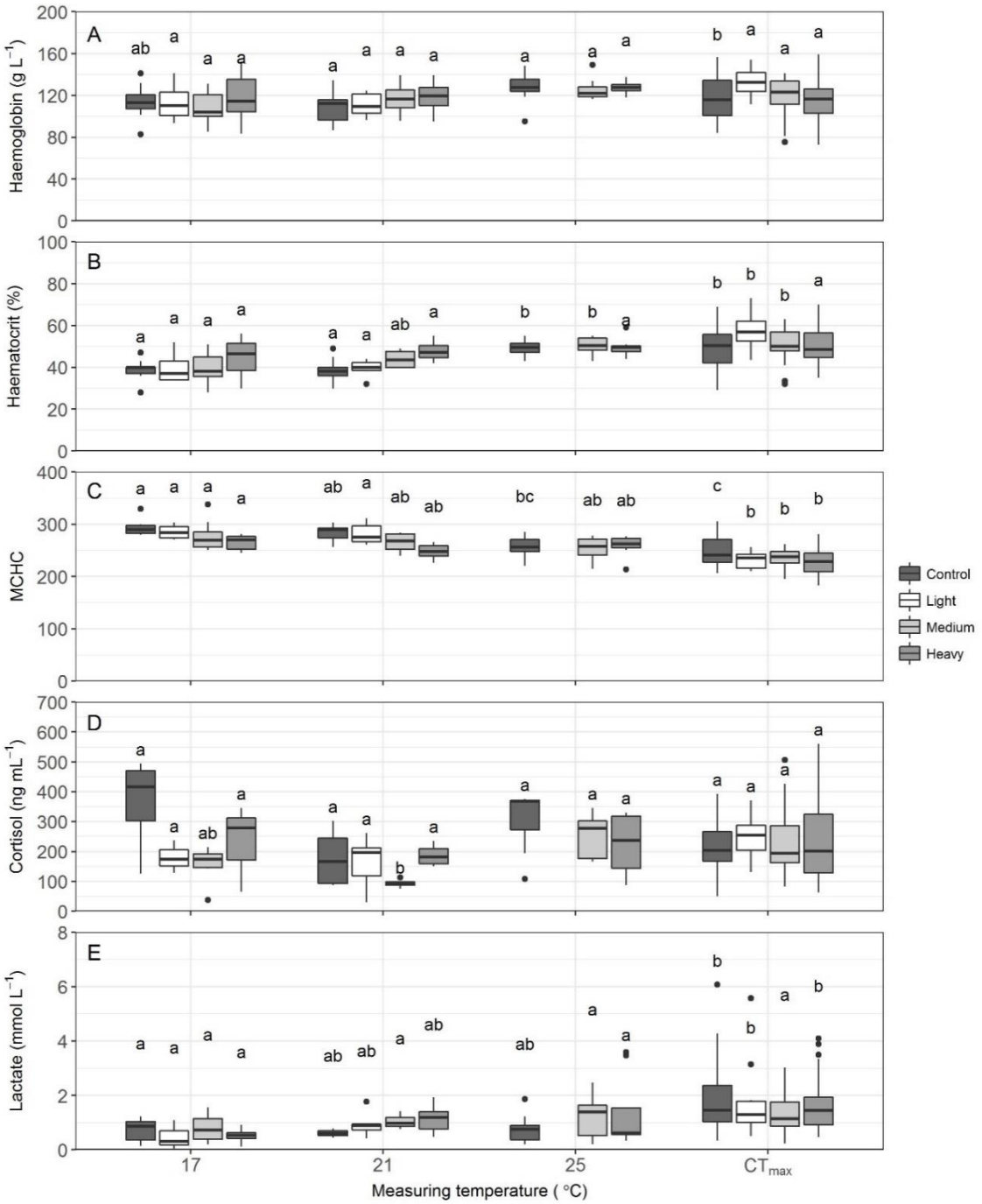

Figure 3.3: (A) Haemoglobin, (B) haematocrit, (C) mean corpuscular haemoglobin concentration, (D) plasma cortisol, and (E) plasma lactate levels in control and AGD-infected Atlantic salmon across the CT_{max} protocol. Boxes represent the 25th quartile, median, and 75th quartile with the whiskers representing the minimum and maximum values. Points depict outliers. Different lowercase letters demarcate significant differences within an infection level across temperatures (letters excluded if no differences exist). Due to the random sampling method at each temperature, no individuals of light infection were sampled at 25°C.

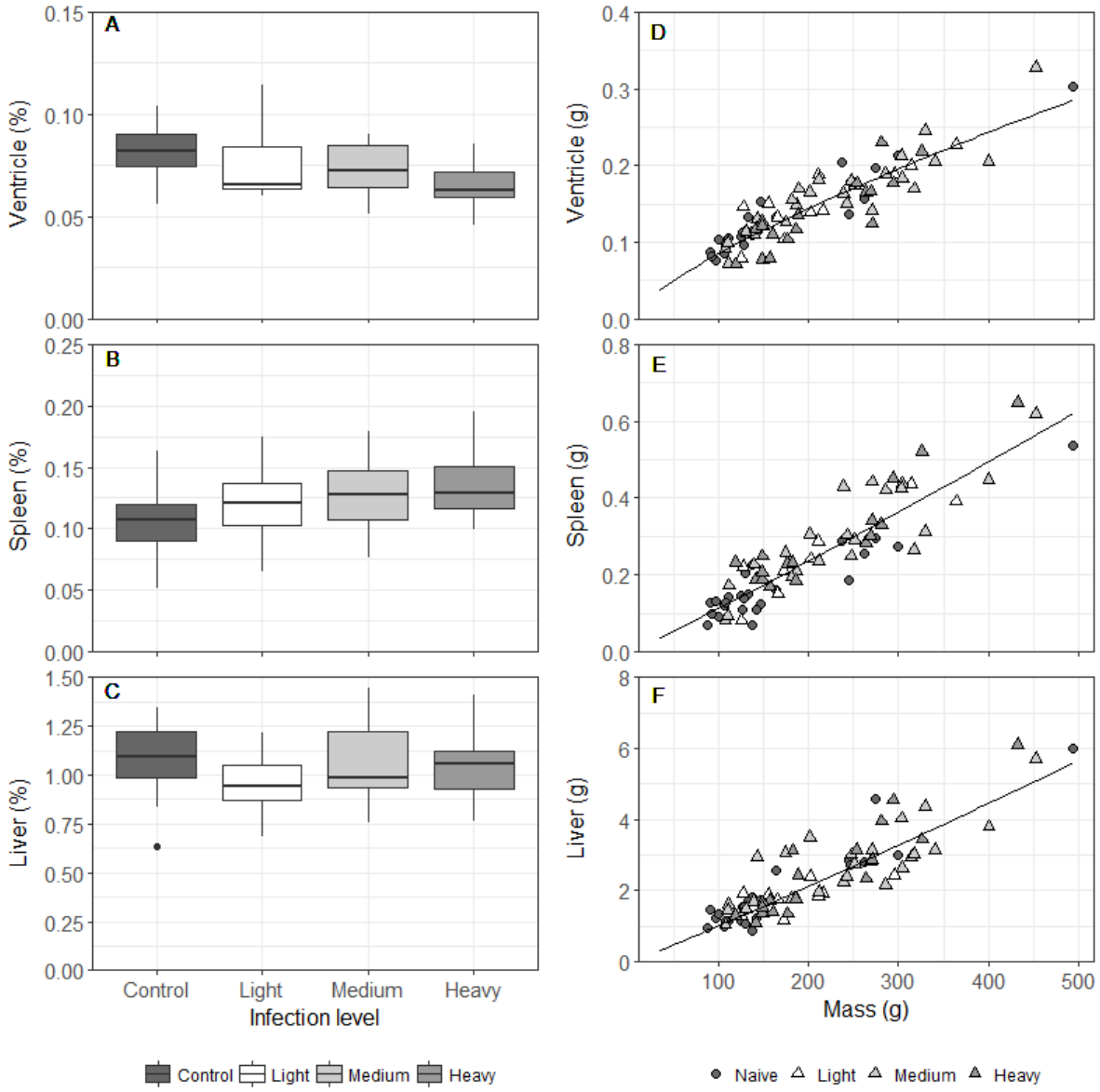

Figure 3.4: (A-C) Ventricle, liver, and spleen masses presented as percent of body mass across infection levels. Boxes represent the 25th quartile, median, and 75th quartile with the whiskers representing the minimum and maximum values. Points represent outliers. No significant differences were found between infection levels. (D-F) Absolute relationships between body mass and organ mass in Atlantic salmon. Data points represent individual fish. Absolute mass regression lines (with standard errors in parentheses) are described by: (D) ventricle mass= 0.003(0.239)* $M_b^{0.758(0.045)}$ (R^2=0.783, p<0.0001); (E) spleen mass= 0.0008(0.070)* $M_b^{1.120(0.070)}$ (R^2=0.775, p<0.0001); (F) liver mass= 0.007(0.456)*$M_b^{1.070(0.086)}$ (R^2=0.672, p<0.0001).

Discussion

The level of AGD infection significantly influenced the thermal tolerance of Atlantic salmon in this study, however it was not the linear relationship that was hypothesized. As expected, fish that exhibited higher AGD loads (gill scores 4 and 5) had a lower CT_{max} than those that were less infected (each of the gill scores 1 to 3). However, while heavily infected fish exhibited a lower thermal tolerance than larger control fish, smaller control fish exhibited similar CT_{max} values as the most heavily infected individuals. There were also higher mortalities of control fish during the overnight settling period in the experimental tanks. Mortality in these fish was likely multi-faceted through a combination of stressors. Firstly, 'weak' (mainly smaller) fish were selected against in the infection stock tanks such that only 'strong' (mainly larger) fish were left for the CT_{max} trials, while there was no selection pressure on the weak/small individuals in the control stock tank so they survived until the time of the CT_{max} trials. This is likely to be a key driver behind the significant breakpoint mass determined for CT_{max} within the control group. Secondly, the use of a common sump within the experimental tank setup resulted in the control fish being exposed to amoebae for the first time and thus suffering higher immediate mortality despite no visible signs of AGD on their gills. The combination of stressors for the 'weaker' fish (e.g. possible malnourishment, deformed, immune suppressed, not quite developed for smolting, etc) along with first exposure to amoebae through the common sump led to the higher mortality observed in the control experimental tank. More than likely, these 'weaker' fish had already succumbed to the disease in the infection tanks as part of the 29% mortality during disease progression. This is unsurprising as runts in a population have been observed to succumb within 24 h of disease exposure. Furthermore, the smaller control fish in this study exhibited similar CT_{max} values to heavily infected fish which were larger on average (131.93 ± 4.56 g compared to 218.31 ± 9.32, respectively). This lends support to the idea that the smaller control fish may be susceptible to multiple stressors in the experimental set-up. This phenomenon was a methodological consideration that was not anticipated to influence the findings, but it has inadvertently highlighted the speed at which the amoeba may compromise survival of the host. In light of these findings, complete separation of experimental tank systems is suggested for even short-term AGD studies in the future.

The CT_{max} values reported here (23.1 to 29.0°C) are lower than previously reported values of 32.6 ± 0.81°C and 29.9 ± 0.79°C for wild Atlantic salmon and brown trout, *Salmo trutta* L., respectively, at similar acclimation temperatures and heating rates albeit in freshwater (Elliott and Elliott, 1995). However, the values reported in this study are similar to those reported for Atlantic salmon in seawater (~26.5°C) using similar heating rate, but the fish were almost three times as large (>600 g)

and acclimated to 10°C (Penney et al., 2014). The lower values in this study compared to the former may be a result of my fish being larger in general (i.e., ~220 g here vs. 2 to 39 g in Elliott and Elliott, 1995), and/or due to differences in thermal tolerance between wild versus domesticated strains of fish. Indeed, a growing number of studies have shown that smaller individuals have higher thermal tolerance than larger conspecifics (Clark et al., 2008a; Pörtner et al., 2008; Daufresne et al., 2009; Clark et al., 2017). Furthermore, CT_{max} was reported to be significantly higher (by ~2°C) in wild versus domesticated strains of brown trout, brook trout, *Salvelinus fontinalis* (Mitchill), and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Carline and Machung, 2001).

While a previous lab-based infection study suggested that temperatures above 16°C drastically increase AGD-related mortalities in Atlantic salmon (Douglas-Helders et al., 2001), this is the first study to quantify a reduction in thermal tolerance of heavily infected individuals. Here, it was demonstrated that light and moderately infected individuals have uncompromised thermal tolerance, but high infection levels lead to reduced CT_{max}. While the difference in CT_{max} values was ~2°C between light and moderate versus heavily infected individuals, the reduction in thermal tolerance could still be detrimental to the aquaculture stock as heat waves, and therefore the corresponding spike in sea surface temperature, become more prevalent in the future (Kirtman et al., 2013). The sea pens of aquaculture facilities keep the fish enclosed within the surface waters and do not allow for behavioural adjustments to cope with the elevated temperatures such as seeking cooler water. Furthermore, the lowered thermal tolerance of heavily infected fish indicates compromised physiology which could be exacerbated during routine stressors due to standard aquaculture practices (e.g. handling, gill scoring, freshwater bathing).

Temperature, rather than AGD infection level, was the primary driver of differences in the blood oxygen transport parameters measured here. No differences have been reported in haemoglobin and haematocrit between AGD-infected and control Atlantic salmon during a serial sampling protocol at 16°C (Leef et al., 2005a). However, a recent study reported lower haemoglobin and haematocrit values in AGD-infected fish compared to their uninfected counterparts (Hvas et al., 2017a). Overall, in this study, haemoglobin remained stable over the CT_{max} protocol with significant differences detected only among lightly infected fish between 21°C and their CT_{max}. Haematocrit increased from 21 to 25°C in all infection levels except for heavily infected fish. Combined with a general decrease in MCHC across temperatures, these findings corroborate reports for other species and are consistent with erythrocyte swelling (Gollock et al., 2006). Erythrocyte swelling is one of three mechanisms (along with splenic release of erythrocytes and loss of plasma water to the tissues) to increase the oxygen carrying capacity of the blood (Wood and Perry, 1985).

Haemoglobin in swollen erythrocytes is thought to have a higher oxygen affinity due to increased intracellular pH, consequently aiding the binding of oxygen under stressful conditions (Soivio and Tuurala, 1981; Milligan and Wood, 1987). The evidence for erythrocyte swelling was not dependent on infection level. The repeated tank disturbances due to the sampling protocol could have influenced the erythrocyte swelling, however, this is unlikely as a gradual increase in cortisol and lactate would have been expected but which was not observed. Therefore, the erythrocyte swelling is suggested to be a product of the temperature increase. Despite the negligible impact of AGD infection on blood oxygen carrying capacity, some evidence was found for a reduction in the ventricle mass of heavily infected fish, which might make for a fruitful direction of future study.

Plasma cortisol concentrations were not influenced by infection level or the sampling temperature, except in the medium infection group (Fig. **3.3**D). Interestingly, plasma cortisol levels were generally elevated compared with resting cortisol concentrations reported previously for juvenile salmonids (0 to 30 ng mL^{-1}) (Wedemeyer et al., 1990; Barton and Iwama, 1991). The elevated cortisol levels seen in this study could be due to various aspects. Many natural and anthropogenic factors influence circulating corticosteroid concentrations in fish such as temperature, nutrition, time of day, disease, psychological stress, and many aquaculture practices (e.g. handling, crowding, etc.) (Robertson et al., 1987; Wedemeyer et al., 1990; Barton and Iwama, 1991; Pankhurst and Dedual, 1994). The fish also only had 12 h to settle after handling/transport from the holding to experimental tanks. Rainbow trout (~89 g), brook trout (~135 g), brown trout (~99 g), and lake trout (~23 g) subjected to 30 s of air exposure exhibited spikes in cortisol directly prior to the stressor and was almost returned to baseline values by 6 h (Barton 2011). While fish in this study are larger on average than those presented in Barton (2011), cortisol levels have been observed as similar independent of fish size during an exhaustive swim protocol (Clark et al 2012). Therefore, it would be expected that 12 h would be sufficient for cortisol levels to return close to resting levels, however this was not seen indicating other stressors at work. The presence of amoebae in the experimental tanks due to the common sump in the experimental set-up may be at least partly responsible for the elevated plasma cortisol concentrations throughout the warming protocol. Support for this idea stems from a report of elevated cortisol concentrations from 0.01 to 276.5 ng mL^{-1} 24 hours after the first signs of a bacterial infection in red drum, *Scianeops ocellatus* (L) (Robertson et al., 1987). The higher overnight mortalities in the experimental tank and a similar thermal tolerance of smaller control to heavily infected fish may indicate a similar stress level, which resulted in similar cortisol levels between control and infected fish.

As a consequence of AGD-associated lesions, it was expected that infected individuals in the present study may utilise anaerobic metabolism to a greater extent than control fish during the warming protocol and thus maintain elevated levels of lactate. However, plasma lactate increased consistently in all infection levels (Fig. **3.3**E). Similarly, no differences in plasma lactate concentrations were reported in Atlantic salmon exposed to AGD at a constant temperature of ~15°C compared to control fish that were exposed to sterile seawater (Leef et al., 2005a). Rainbow trout can recruit previously unperfused lamellae in order to maintain aerobic respiration in challenging environments, such as high temperature or hypoxia (Booth, 1979). Thus, it is possible that lamellar recruitment was already occurring in heavily infected salmon to compensate for the decrease in functional surface area due to lamellar fusion, subsequently maintaining lactate levels the same as in control fish. Studies examining gill perfusion and oxygen/ion transport capacity would help to address this idea. Furthermore, if metabolism switched from aerobic to anaerobic due to the increased metabolic demand of increasing temperatures, lactate levels would presumably spike at CT_{max} which was not seen in this study. Therefore, these results further support the idea that thermal tolerance is not governed by oxygen limitation (Clark et al., 2013).

Overall, heavily infected Atlantic salmon were found to have a reduced thermal tolerance compared to their lightly infected conspecifics. Currently, AGD is controlled on farms by bathing fish in freshwater for 2 to 4 h once a certain percentage of the stock reaches gill scores of 2 to 3 (Taylor et al., 2009b). Thus, while current bathing practices may be suitable to maintain low gill scores and prevent any influence of AGD on thermal tolerance of the stock, more research is required to quantify any decrements in other performance metrics at lower gill scores (e.g. digestive efficiency, growth, chronic temperature tolerance). Moreover, almost nothing is known about AGD in the context of multiple, interactive stressors like temperature, salinity, hypoxia and handling, all of which are important factors in the aquaculture environment.

Chapter 4: Amoebic gill disease increases energy requirements and decreases hypoxia tolerance in Atlantic salmon (*Salmo salar*) smolts

Abstract

Atlantic salmon (*Salmo salar* Linnaeus) in the Tasmanian aquaculture industry are routinely affected in summer months by an ectoparasitic amoeba that attaches to the gills and causes 'amoebic gill disease' (AGD). The disease has been implicated in decreasing the tolerance of salmon to environmental perturbations like heatwaves and hypoxia, yet little empirical evidence exists to support these anecdotal observations. Using groups of fish acclimated to 15 or 19°C, my aim was to determine the effects of industry-relevant levels of AGD on resting and maximal metabolic rates ($\dot{M}O_{2rest}$ and $\dot{M}O_{2max}$, respectively), aerobic scope ($\dot{M}O_{2max} - \dot{M}O_{2rest}$), recovery from anaerobic exercise (excess post-exercise oxygen consumption [EPOC]), and hypoxia tolerance (critical oxygen tension [P_{crit}] and dissolved oxygen level at loss of equilibrium [DO at LOE]). Interestingly, there was no interaction between acclimation temperature and AGD infection level for any of the measured parameters, suggesting similar impacts of the disease within each temperature. Within both acclimation temperatures, an increase in $\dot{M}O_{2rest}$ (~8% and ~13% increase within the 15 and 19°C acclimation groups, respectively) with increasing AGD infection levels demonstrated a progressive increase in baseline energy requirements as the disease progressed. On the other hand, $\dot{M}O_{2max}$ remained stable across the infection levels at both temperatures (~364 mg O_2 kg^{-1} h^{-1}), resulting in a decline in aerobic scope by 13 and 19% in the 15 and 19°C groups, respectively, with disease progression. Neither EPOC nor P_{crit} were influenced by the infection within either temperature, yet there was evidence of a decrease in hypoxia tolerance since DO at LOE increased with increasing infection levels. These results suggest an increase in energy requirements and a reduction in whole-animal performance as AGD proliferates, lending support to idea that AGD reduces environmental tolerance. However, the lack of an effect of acclimation temperature in this study indicates that the temperature-disease interaction may be more complicated than currently thought.

Introduction

Amoebic gill disease (AGD) is a predominant health issue facing the Atlantic salmon (*Salmo salar* Linnaeus) aquaculture industry in Tasmania, Australia (Roubal et al., 1989; Munday et al., 1990; Nowak, 2001). As stated previously in the General Introduction, temperature is a key risk factor in AGD outbreaks, caused by *Paramoeba perurans*, resulting in a proliferation of the disease in summer months and particularly when seawater temperatures exceed ~16˚C (Munday et al., 1990; Nowak, 2001). Thus, the above-average rate of temperature increase in Tasmanian waters represents a primary concern for the health of fish in the Atlantic salmon aquaculture industry (Popova et al., 2016).

Any damage to gill tissue, through the hyperplastic lesions, can have deleterious consequences on whole animal performance because the gills act as a primary site for oxygen uptake and ionoregulation (McDonald, 1983; Rombough and Ure, 1991; Wells and Pinder, 1996). Thus, because the amoebae attach and cause damage to the gills, it is plausible that AGD-affected fish will suffer from respiratory distress and decreased hypoxia tolerance. Some support for this idea stems from observations of infected individuals exhibiting lethargy and rapid ventilation prior to mortality (Munday et al., 1990).

The few studies that have examined oxygen uptake rates ($\dot{M}O_2$) in AGD-infected fish have yielded inconclusive and/or conflicting results (Powell et al., 2000; Fisk et al., 2002; Leef et al., 2005a; Leef et al., 2007a; Leef et al., 2007c). For example, there were negligible differences in the $\dot{M}O_2$ of control and AGD-infected Atlantic salmon sourced from commercial sea pens under normoxic conditions and at an ambient temperature of ~17˚C (Powell et al., 2000; Fisk et al., 2002). However, two studies of juvenile Atlantic salmon reported an increasing trend in routine $\dot{M}O_2$ after 48 h of amoebae exposure in normoxia at ~15.5˚C (Powell et al., 2005; Leef et al., 2007c). The influence of AGD on maximum $\dot{M}O_2$ has received little attention despite its potential to provide insight into oxygen transport capacity limitations that may be caused by reduced gill surface area associated with AGD-related fusion of secondary lamellae. Nevertheless, in the only relevant studies of which the authors are aware, AGD-infected Atlantic salmon in normoxia achieved the same maximum $\dot{M}O_2$ following a burst exercise protocol as their non-infected counterparts (Powell et al., 2005; Leef et al., 2007c). These results are contrary to a more recent study that utilised a critical swimming speed test and observed a lowered $\dot{M}O_{2max}$ in AGD-infected Atlantic salmon compared to the controls (Hvas et al., 2017a). Notably, all studies to date have investigated AGD-induced respiratory effects at a single temperature of either 13˚C,

~15.5˚C, or ~17˚C (Powell et al., 2000; Fisk et al., 2002; Powell et al., 2005; Leef et al., 2007c). Summer temperatures exceeding 17˚C are associated with the greatest proliferation of the disease and therefore may be more relevant for examining how AGD affects respiratory physiology (Kent et al., 1988).

In addition to potential detrimental effects on $\dot{M}O_2$, AGD lesions can also effect processes such as excess post-exercise oxygen consumption (EPOC) or hypoxia tolerance, typically measured as the critical oxygen tension (P_{crit}). Due to the compromised gill surface area in AGD-infected fish, a greater reliance on anaerobic respiration could occur during exhaustive exercise. Furthermore, hypoxia tolerance could be lowered if anaerobic respiration is utilised to a greater extent which would translate into a higher P_{crit} (the oxygen tension below which resting $\dot{M}O_2$ can no longer be maintained.

Here, a comprehensive examination is presented of how AGD affects the respiratory physiology of Atlantic salmon across a summer temperature range. Specifically, resting $\dot{M}O_2$, maximum $\dot{M}O_2$, and aerobic scope of AGD-infected versus control Atlantic salmon were measured at two acclimation temperatures (15 and 19˚C). Furthermore, this study provides the first investigation of whether AGD affects EPOC or P_{crit}. Given the well-established effect of temperature on increasing the aerobic metabolic requirements of fishes, it was hypothesized that any signs of respiratory distress and anaerobic metabolism associated with AGD will be more severe in fish acclimated to the warmer treatment temperature (Clarke and Johnston, 1999; Gillooly et al., 2001; Clark et al., 2013). The objective is to elucidate whether a limitation in aerobic capacity may be the driver of lower performance of AGD-infected salmon at elevated temperatures and when exposed to hypoxia.

Methods

Fish husbandry and acclimation

Atlantic salmon parr (n=210; mass=~87 g) were transported from the SALTAS Hatchery in Wayatinah, Tasmania to the Aquaculture Centre at the University of Tasmania in Launceston where all experiments were conducted. Fish were held in 3500 L freshwater recirculation tanks for four weeks to recover from transport at 14°C. The recirculation system was equipped with solids filtration, bio-filtration and UV disinfection. Water parameters were maintained at > 90% dissolved oxygen, < 1 mg L^{-1} TA-N, < 0.5 mg L^{-1} NO_2^-, < 80 mg L^{-1} NO_3^{2-} and pH 7.0 – 7.2. Water was exchanged at ~10% per day. Fish were fed to satiation daily to promote growth. Once the fish were ~ 150 g, they were separated randomly into 8 x 300 L

independently-recirculated freshwater tanks (n=24 to 26 per tank) and held at 15°C for a further two weeks in temperature-controlled rooms (4 tanks per room). Thereafter, salinity and temperature were incrementally increased over three weeks, to reach full strength seawater (35 psu) and the desired acclimation temperatures (15 and 19°C) and held there for a further four weeks before infection. The increase in salinity was achieved by exchanging full strength seawater every two to three days, whereas the increase in temperature was achieved by modifying the set-point temperature of the rooms. Water parameters during the acclimation and infection periods were maintained at > 90% dissolved oxygen, < 2 mg L^{-1} TA-N, < 5 mg L^{-1} NO_2^-, < 160 mg L^{-1} NO_3^{2-} and pH 8.0 – 8.2. Each recirculation system was equipped with solids filtration, foam fractionation, bio-filtration and UV disinfection.

Infection protocol

The infection protocol was similar to that of Morrison et al. (2004). Briefly, post-mortem AGD-infected Atlantic salmon were sourced from an on-going AGD trial. Gills were excised, the arches separated in distilled water and gently agitated for 2 min. The debris was transferred to an equal volume of double strength seawater, briefly mixed and then placed onto plastic Petri dishes (~ 20 ml of solution) and the amoebae allowed to adhere for 1 h at 18°C. Then the debris was discarded and the Petri dishes washed three times with 0.2 µm filtered seawater to harvest the amoebae. The adherent cells were dislodged with 2.5 ml of distilled water, a further 2.5 ml of double strength seawater was added and the cells and water were transferred to falcon tubes (50 ml) and amoebae enumerated with a haemocytometer.

The recirculation systems of the tanks to be infected (two tanks at 15˚C and two at 19˚C) were turned off, and drained to a volume of 80 L to produce systems of aerated static seawater. Amoebae were introduced into the tanks at 531 cells L^{-1} and fish were monitored for 6 h. Then the recirculation system was turned back on and any remaining free-floating amoeba were removed by the filtration system. The infection was allowed to progress for three weeks prior to experiments commencing.

Experimental set-up

Two water baths in each temperature-controlled room (one for control fish and one for AGD-infected fish) each housed four respirometers (three 7 L and one 5 L). Water temperature was controlled passively through room temperature (15 and 19°C). Each

respirometer contained a small recirculation pump (~3 L min^{-1}) to ensure adequate water mixing. Flush pumps were connected to two respirometers each to intermittently flush (~7 L min^{-1} $respirometer^{-1}$) the respirometers with clean, aerated water on a 10-min cycle (5 min seal and 5 min flush). Oxygen concentration was measured in each respirometer every 5 s by fibre optic probes positioned near the recirculation pump and connected to a four channel FireSting O_2 Optical Oxygen Meter linked with a PC running FireSting software (Pyroscience, Aachen, Germany).

Experimental protocol

Fish that were fasted for 24 h were placed in respirometers in the water baths corresponding to their respective acclimation temperature (15 or 19˚C) and treatment (AGD or control) in the evening and allowed to acclimate overnight (>12 h) during which resting $\dot{M}O_2$ ($\dot{M}O_{2rest}$) was measured. The following morning, fish were individually removed from their respirometers and exercised in a 40 L round tank at their acclimation temperature. Preliminary investigations showed that 90% of fish chased exhausted by 2 min. Therefore, each fish was chased for 2 min by hand and then immediately placed back into their original respirometer to record maximum $\dot{M}O_2$ ($\dot{M}O_{2max}$). A 'swim score' was assigned to each fish based on the time (in 10 s increments) during the chase at which the fish stopped frequently bursting and the chaser could grab the tail (e.g., 1 to 10 s= 1; 11 to 20 s= 2; 21 to 30 s= 3, etc.). Each day, all control fish were exercised prior to AGD-exposed fish to prevent cross-contamination of amoeba. Fish were left undisturbed on the 5:5 min seal:flush cycle for 4 h post-exercise to measure the excess post-exercise oxygen consumption (EPOC). The respirometers were then sealed such that oxygen levels declined (due to fish respiration) for the determination of the critical oxygen tension (P_{crit}). The P_{crit} test was terminated when the fish lost equilibrium (LOE) for at least 5 s, at which point the oxygen level was recorded (termed 'DO at LOE' herein). Each fish was then removed from the respirometer and euthanised before mass and fork length were measured. Blood was extracted via caudal puncture (4 mL lithium-heparinised vacutainers and 22 G needles) and put on ice (<1 h) for further processing (see below). After all fish reached LOE and were removed from respirometers, the respirometers were resealed to quantify background respiration for at least 30 min. All equipment was thoroughly drained and cleaned before the water was replenished for the next run.

Following each LOE test, the heart of each fish was dissected out and the ventricle was

separated from the atrium and bulbus prior to being squeezed free of blood, weighed, and then fixed in 70% ethanol. The gill basket of each fish was also extracted, washed in seawater, and placed in Davidson's seawater fixative. Photographs of each hemibranch were taken within 48 h and then the hemibranchs were placed in 70% ethanol for long term storage. Experiments were run over the course of two weeks to result in a total of 48 infected fish and 15 to 16 control fish at each temperature (see Table 4.1).

Data analyses and statistics

Condition factor was calculated from body mass and length using Eq. 4.1:

(4.1) $\text{Condition factor}=100M_b/L^3$

where M_b is body mass (g) and L is fork length (cm) (Fulton 1904). Fish with a condition factor less than 0.7 (totalling 3% of fish) were deemed unhealthy according to salmonid industry guidelines and thus they were omitted from subsequent analyses (Acharya 2011). Mass, length and condition factor were not correlated with percent lesion coverage on the gills, so data for these parameters were analysed using two-way ANOVAs to test the parameter against infection status (control versus AGD-infected) and temperature acclimation group (15 and 19˚C).

Resting and maximum oxygen consumption rates (mg O_2 kg^{-1} h^{-1}) were calculated using Eq. 2.2. Resting metabolic rate ($\dot{M}O_{2rest}$) was determined as the mean of the lowest 10% of oxygen consumption values throughout the measuring period, excluding outliers (values ± 2 s.d. from the mean (Norin et al., 2014)). Maximum metabolic rate ($\dot{M}O_{2max}$) was calculated from a 3-min slope immediately after the exhaustive chase protocol, which was always found to be the highest. Absolute aerobic scope was calculated by subtracting $\dot{M}O_{2rest}$ from $\dot{M}O_{2max}$, while factorial aerobic scope was calculated by dividing $\dot{M}O_{2max}$ by $\dot{M}O_{2rest}$.

Excess post-exercise concentration (EPOC) was quantified by finding the area under the curve between $\dot{M}O_{2max}$ and $\dot{M}O_{2rest}$. This was achieved by modifying the SDA (specific dynamic action) code in the fishMO2 R package (Chabot, 2016; Claireaux and Chabot, 2016; Chabot et al., 2016). In brief, the function calculates the area under a curve fitted with an rqss regression. The user must specify a list of times (in hours) and associated $\dot{M}O_2$ values with time 0 h corresponding with peak $\dot{M}O_2$ (in this study $\dot{M}O_{2max}$) as well as a tolerance value (typically 0.05, meaning 5%) that is the value added to $\dot{M}O_{2rest}$ to

determine the end of the function. The function also calculates EPOC duration (the length of time it takes for $\dot{M}O_2$ to reach $\dot{M}O_{2rest}$). If $\dot{M}O_{2rest}$ was not reached within the allotted time, the function extrapolates the curve down to the user-specified $\dot{M}O_{2rest}$ (plus 5% based on the specified tolerance value).

Critical oxygen tension (P_{crit}) was calculated using the calcO2crit function from the fishMO2 R package (Chabot, 2016; Claireaux and Chabot, 2016). The function fits a linear regression through the $\dot{M}O_2$ values below the pivotal DO value (the DO value where $\dot{M}O_2$ is lowest above the fifth percentile of all $\dot{M}O_2$ values). P_{crit} is then determined as the DO level where the linear regression line intercepts $\dot{M}O_{2rest}$.

Haemoglobin concentration ([Hb]) was measured in each blood sample using a HemoCue™ haemoglobin analyser (HemoCue 201$^+$, Angelholm, Sweden). [Hb] values were corrected to that of salmon using Eq. 4.2:

(4.2) $[Hb] = 0.820\ x - 5.831$

from Andrewartha et al. (2016), since the HemoCue is designed to measure human [Hb] (Clark et al., 2008b). Haematocrit (Hct) was measured using 16 µL of whole blood spun at 11,000 rpm for 1 min (SpinCrit Microhematocrit Centrifuge, USA). Subsequently, mean corpuscular haemoglobin concentration (MCHC) was calculated from [Hb] and Hct values using Eq. 4.3:

(4.3) MCHC= [Hb]/(Hct/100)

Gill images were analysed in ImageJ using a similar method to that described previously (Pennacchi et al., 2016). Briefly, the images were sharpened and the total gill filament area was measured as the total hemibranch surface area minus the area of the gill arch. Then, the area was determined for each focal white spot (lesions associated with AGD). The total percent lesion coverage of the affected gill area was calculated using Eq. 4.4:

(4.4) % lesion coverage = (total lesion area / total gill filament area)*100

where the total lesion area is the sum of all the lesion areas present on all hemibranchs for an individual, and the total gill filament area is the sum of the filament areas for all 16 hemibranchs of an individual.

$\dot{M}O_{2rest}$, $\dot{M}O_{2max}$, absolute aerobic scope ($\dot{M}O_{2max}$ - $\dot{M}O_{2rest}$), factorial aerobic scope ($\dot{M}O_{2max}$ /

$\dot{M}O_{2rest}$), EPOC, P_{crit}, DO at LOE and ventricle mass were analysed using two-way ANCOVAs (Type III sums of squares) with mass as a covariate, percent lesion coverage as a continuous predictor, and acclimation temperature as a categorical predictor. Swim score was investigated for influence on $\dot{M}O_{2max}$, but it was determined to not affect $\dot{M}O_{2max}$ so was disregarded from the final model. For other variables (EPOC duration, haemoglobin, haematocrit, and MCHC), mass did not significantly improve the models so it was dropped as a covariate and two-way ANOVA models were used with infection status and acclimation temperature as predictor variables. Data are presented herein as mass-specific $\dot{M}O_2$ (mg kg^{-1} h^{-1}). Data were log-transformed where applicable to satisfy statistical assumptions. All statistical analyses were conducted using R Studio (version 1.0.143) using R package *car* (Fox and Weisberg, 2011).

Results

Mass, fork length and condition factor were similar between temperatures (Table 4.1; mass: $F_{1,123}$=0.00, p=0.957; length: $F_{1,123}$=0.00, p=0.963; condition factor: $F_{1,123}$=0.02, p=0.895) and infection groups (Table 4.1; $F_{1,123}$=0.60, p=0.439; length: $F_{1,123}$=2.05, p=0.155; condition factor: $F_{1,123}$=0.19, p=0.665). Fish across all groups were 222.48 ± 4.75 g and 270.70 ± 1.76 mm with a condition factor of 1.10 ± 0.01 (see Table 4.1**Error! Reference source not found.**).

Table 4.1: Sample sizes, mass, length and condition factor for AGD-infected and control Atlantic salmon acclimated to two temperatures.

Treatment	Acclimation temperature (°C)	N	Mass (g)	Length (mm)	Condition factor
AGD	15	48	220.44 ± 6.90	271.19 ± 2.42	1.09 ± 0.02
Control	15	15	222.22 ± 11.58	270.33 ± 4.69	1.12 ± 0.03
AGD	19	48	225.92 ± 7.86	273.69 ± 2.64	1.09 ± 0.03
Control	19	16	207.34 ± 19.20	263.25 ± 7.38	1.12 ± 0.08

Resting $\dot{M}O_2$ was consistently ~14% higher within the 19°C acclimation group (Fig. 4.1A; $F_{1,88}$=11.12, p=0.001). Resting $\dot{M}O_2$ increased with lesion coverage from lightly infected fish (0 to 1% lesion coverage) through to the higher AGD loads (>5% lesion coverage), increasing from 76.6 ± 3.29 to 88.9 ± 3.84 mg O_2 kg^{-1} h^{-1} at 15°C and from 96.7 ± 7.16 to 103.5 ± 10.27 mg O_2 kg^{-1} h^{-1} at 19°C (Fig. 4.1A; $F_{1,88}$=4.58, p=0.035). There was no significant interaction between percent lesion coverage and acclimation temperature, indicating that percent lesion coverage had the same absolute effect on $\dot{M}O_{2rest}$ at the two temperatures ($F_{1,88}$=0.009, p=0.921). In contrast to $\dot{M}O_{2rest}$, maximum $\dot{M}O_2$ was not influenced by percent lesion coverage or acclimation temperature (367.9 ± 5.26 mg O_2 kg^{-1} h^{-1} for all fish) (Fig. 4.1B; percent lesion coverage: $F_{1,110}$=2.21, p=0.140; acclimation temperature: $F_{1,110}$=0.21, p=0.644). Consequently, absolute aerobic scope was ~12% higher ($F_{1,85}$=1.63, p=0.205) and factorial aerobic scope was ~20% higher ($F_{1,85}$=14.01, p<0.001) in fish acclimated to 15˚C compared with 19˚C. Percent lesion coverage negatively influenced absolute aerobic scope by ~9% ($F_{1,85}$=6.26, p=0.014) and factorial aerobic scope by ~14% ($F_{1,85}$=13.74, p<0.001) as infection level increased (Fig. 4.1C, D).

Neither percent lesion coverage or acclimation temperature influenced EPOC (305.3 ± 9.02 mg O_2 kg^{-1} h^{-1} across all individuals) (Fig. 4.2A; percent lesion coverage: $F_{1,84}$=0.29, p=0.590; acclimation temperature: $F_{1,84}$=0.37, p=0.545). Duration of EPOC, however, was significantly higher in fish acclimated to 15°C compared to 19°C (6.0 h vs. 5.2 h, respectively; $F_{1,85}$=15.24, p<0.001), but was not influenced by percent lesion coverage (Fig. 4.2B; $F_{1,85}$=0.33, p=0.570).

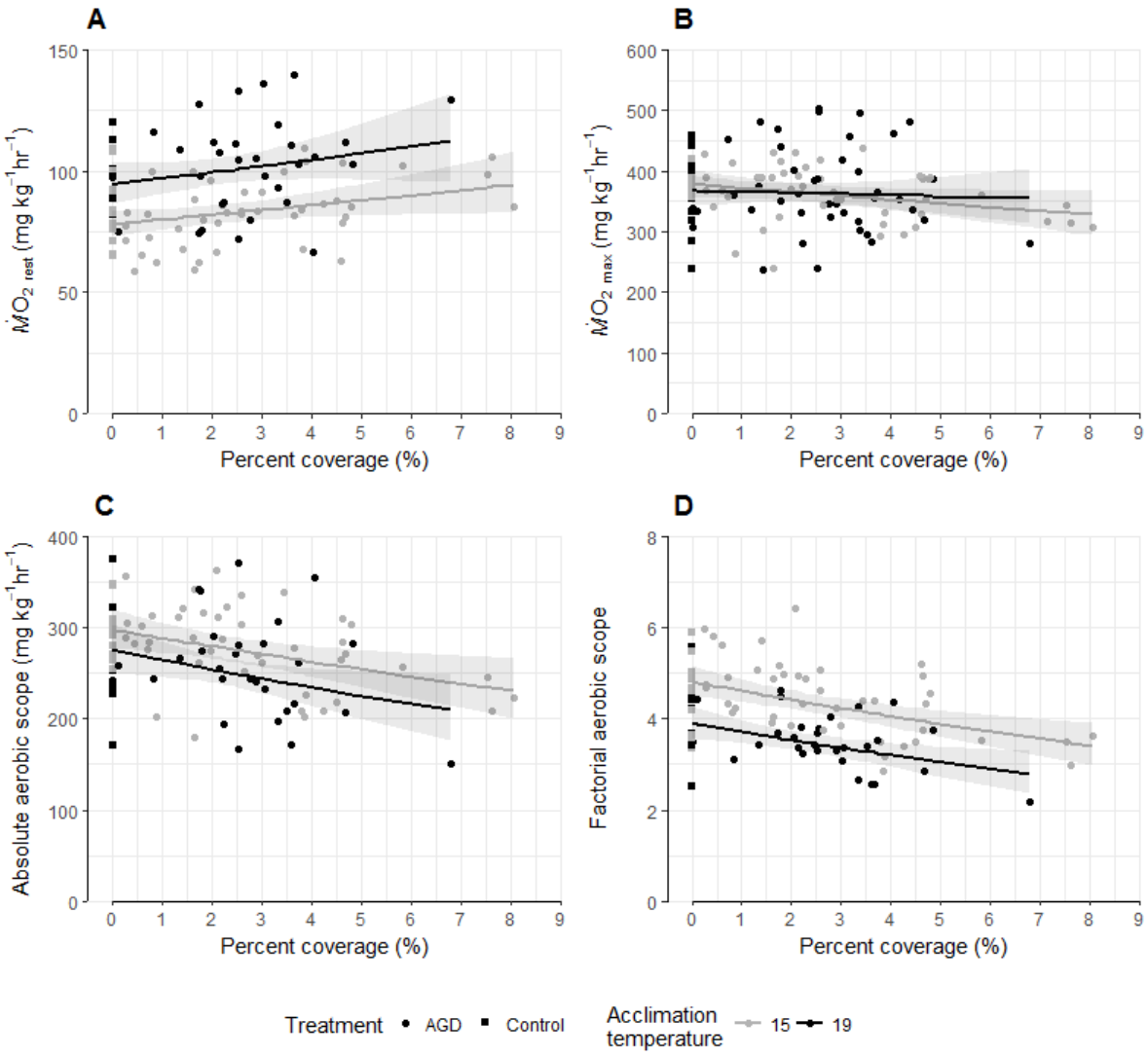

Figure 4.1: (A) Resting oxygen uptake rate, ($\dot{M}O_{2rest}$) (B) maximum oxygen uptake rate ($\dot{M}O_{2max}$), (C) absolute aerobic scope, and (D) factorial aerobic scope across percent coverage of lesions on their gills for AGD-infected (circles) and control (squares) Atlantic salmon individuals acclimated to 15 (grey) and 19°C (black). Bands are 95% confidence intervals and regression lines are described by the equations where x is the percent coverage: (A) 15°C: $\dot{M}O_{2rest} = 78.18e^{0.024x}$; 19°C: $\dot{M}O_{2rest} = 94.51e^{0.238x}$ (B) 15°C: $\dot{M}O_{2max} = 378.33e^{-0.017x}$; 19°C: $\dot{M}O_{2max} = 367.12e^{-0.004x}$ (C) 15°C: Absolute aerobic scope $= 298.26e^{-0.032x}$; 19°C: Absolute aerobic scope $= 276.11e^{-0.041x}$ (D) 15°C: Factorial aerobic scope $= 4.82e^{-0.043x}$; 19°C: Factorial aerobic scope $= 3.91e^{-0.050x}$.

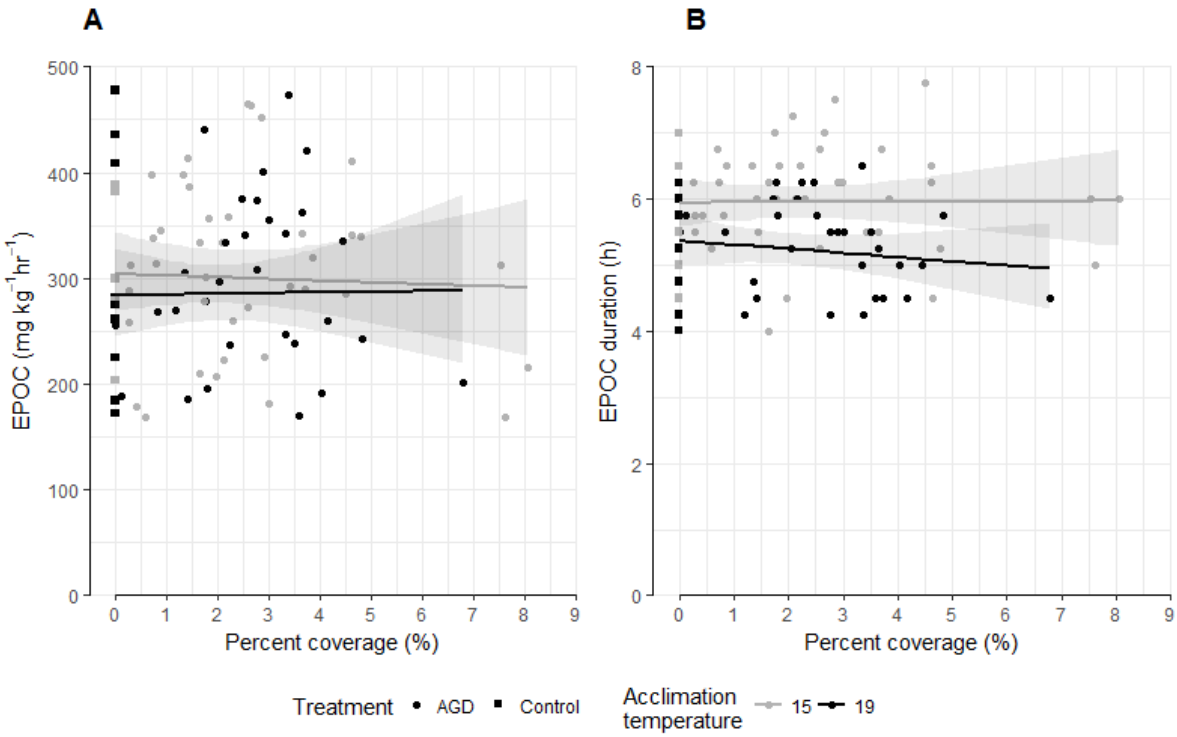

Figure 4.2: (A) Excess post-exercise oxygen uptake (EPOC) and (B) EPOC duration for AGD-infected (circles) and control (squares) Atlantic salmon individuals acclimated to 15 (grey) and 19°C (black) across percent coverage of lesions on their gills. Bands are 95% confidence intervals and regression lines are described by the equations where x is the percent coverage: (A) 15°C: EPOC = $304.05e^{-0.005x}$; 19°C: EPOC = $283.82e^{0.003x}$ (B) 15°C: EPOC duration = $5.94e^{0.001x}$; 19°C: EPOC duration = $5.37e^{-0.012x}$.

While P_{crit} was not influenced by percent lesion coverage at either acclimation temperature (Fig. 4.3A; $F_{1,98}$=1.07, p=0.304), the P_{crit} of fish acclimated to 19°C was ~18% higher than those acclimated to 15°C (31.9 ± 0.76% vs. 26.0 ± 0.45% air saturation, respectively; Fig. 4.3A; $F_{1,98}$=12.93, p<0.001). Similarly, the DO at LOE was ~25% higher in the 19°C acclimation group (Fig. 4.3B; $F_{1,105}$=22.10, p<0.001). There was a significant increase in DO at LOE (i.e., decreased hypoxia tolerance) associated with percent lesion coverage (Fig. 4.3B; $F_{1,105}$=5.06, p=0.027) driven by the fish in the 15°C acclimation group. The DO at LOE in fish at 15°C increased from 15.4 ± 1.31% to 18.2 ± 1.10% air saturation from the lighter (0 to 1% lesion coverage) to heavier (>5% lesion coverage) infected fish, while DO at LOE for fish in the 19°C acclimated group remained stable across percent lesion coverage at 22.4 ± 0.82% air saturation.

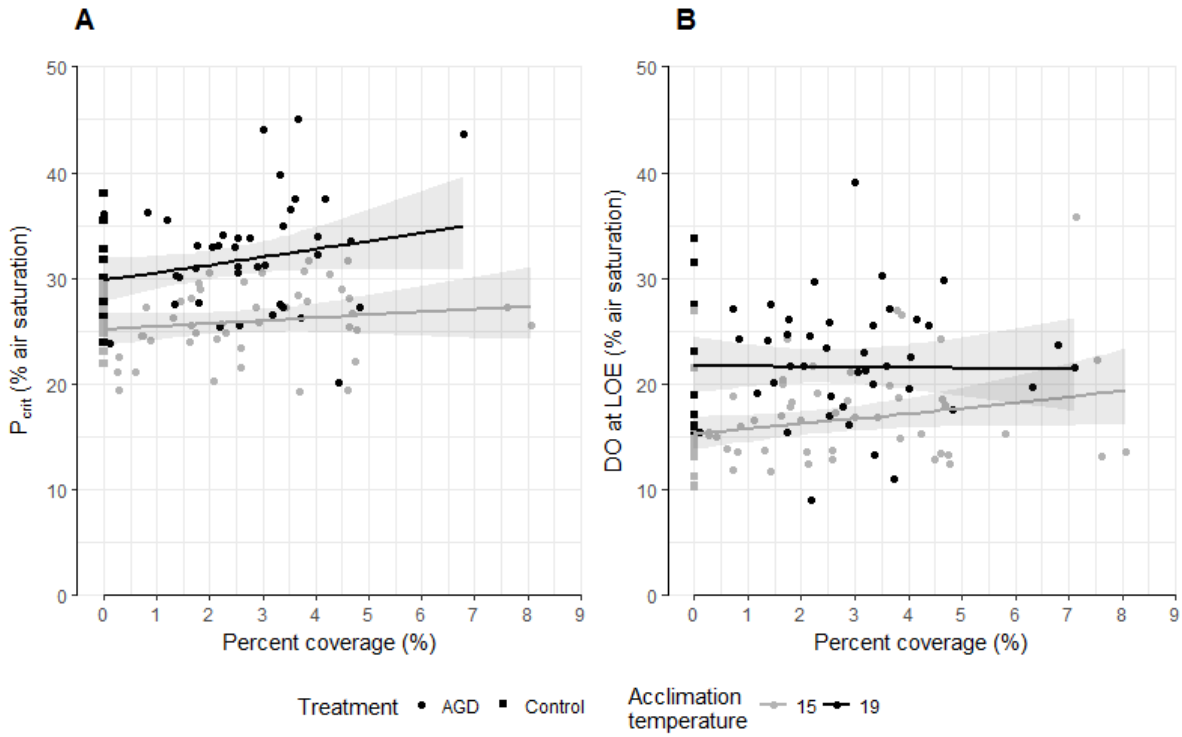

Figure 4.3: (A) Critical oxygen tension (P_{crit}) and (B) DO at LOE of AGD-infected (circles) and control (squares) Atlantic salmon individuals acclimated to 15 (grey) and 19°C (black) across percent coverage of lesions on their gills. Bands are 95% confidence intervals and regression lines are described by the equations where x is the percent coverage: (A) 15°C: $P_{crit} = 25.16e^{0.012x}$; 19°C: $P_{crit} = 29.83e^{0.023x}$ (B) 15°C: DO at LOE = $15.32e^{0.029x}$; 19°C: DO at LOE = $21.70e^{-0.002x}$.

Ventricle mass was not influenced by acclimation temperature (Fig. 4.4; $F_{1,111}$=0.07, p=0.79) or percent lesion coverage, with the relative ventricle mass being 0.08 ± 0.001% of body mass across all individuals (Fig. 4.4; $F_{1,111}$=3.04, p=0.08). Similarly, no haematological measurements were influenced by percent lesion coverage. After pooling the haematological measurements into AGD-infected and control individuals at each acclimation temperature, there were no significant differences in [Hb], Hct or MCHC between infection levels (Table 4.2; [Hb]: $F_{1,113}$=0.47, p=0.495; Hct: $F_{1,114}$=0.88, p=0.349; MCHC: $F_{1,113}$=1.69, p=0.196) or acclimation temperatures (Table 4.2; [Hb]: $F_{1,113}$=1.47, p=0.229; Hct: $F_{1,114}$=1.18, p=0.180; MCHC: $F_{1,113}$=0.02, p=0.881).

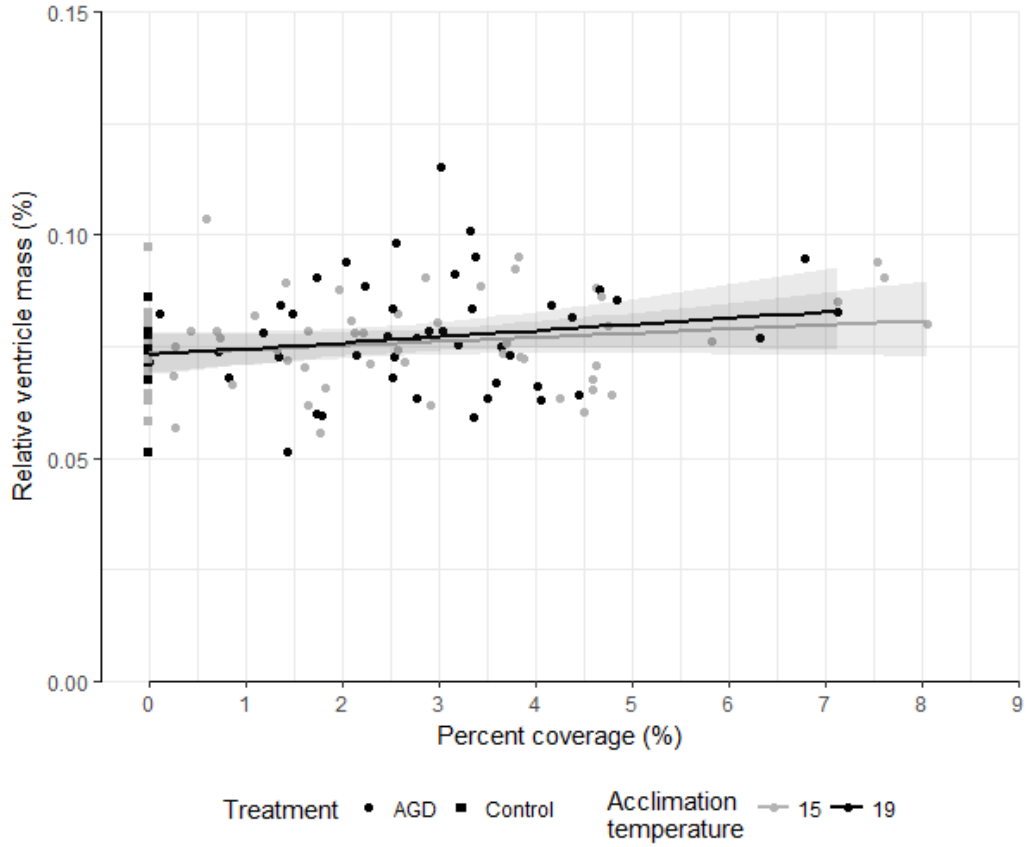

Figure 4.4: Relative ventricle mass for AGD-infected (circles) and control (squares) Atlantic salmon individuals acclimated to 15 (grey) and 19°C (black) across percent coverage of lesions on their gills. Brands are 95% confidence intervals and regression lines are described by the equations where x is the percent coverage: (A) 15°C: Relative ventricle mass = $0.07e^{0.012x}$; 19°C: Relative ventricle mass = $0.07e^{0.018x}$.

Table 4.2: Haematological parameters for control and AGD-exposed Atlantic salmon acclimated to two temperatures.

Treatment	Acclimation temperature (°C)	Haemoglobin ($g\ L^{-1}$)	Haematocrit (%)	MCHC
AGD	15	84.57 ± 1.27	43.02 ± 0.91	199.75 ± 4.9
Control	15	82.41 ± 2.61	41.15 ± 1.60	203.47 ± 8.35
AGD	19	81.14 ± 1.16	41.59 ± 0.79	198.03 ± 4.39
Control	19	86.08 ± 2.05	41.00 ± 1.29	214.11 ± 10.27

Discussion

This is the first study to the author's knowledge that investigates the impacts of AGD on salmon metabolism across a well-characterised continuum of percent gill lesion coverage. Levels of AGD achieved in this study were equivalent to threshold levels (gill score 3) used by the aquaculture industry to initiate a freshwater bathing regime to manage the disease (the controls would correspond to a gill score of 0 whereas a lesion coverage greater than ~3 % would correspond to a gill score of 3) (Taylor et al., 2009a). Thus, results reported here are of relevance to current farm management practices. Percent lesion coverage, both in isolation and in combination with temperature, significantly influenced many of the measured parameters associated with aerobic and anaerobic metabolism. In fact, only $\dot{M}O_{2max}$ and EPOC were not influenced by either percent lesion coverage or acclimation temperature (Figs. 4.1B, 4.2A). The main findings are discussed herein, particularly in the context of potential links between AGD and each of aerobic and anaerobic metabolism. Given that the effect of percent lesion coverage was (unexpectedly) consistent between temperatures, the concentrate is primarily on the effects of AGD rather than discussing temperature in isolation.

Aerobic respiration

The increase in $\dot{M}O_{2rest}$ between 15 and 19°C was not paralleled by a similar increase in $\dot{M}O_{2max}$, which translated to a higher absolute and factorial aerobic scope at 15°C (Fig. 4.1B-D). Since resistance to disease can be dependent upon aerobic scope (Castro et al., 2013; Bruneaux et al., 2017), it could be argued that AGD-infected fish acclimated to 19°C should be compromised compared to those at 15°C because of a lower capacity for increasing oxygen transport. However, the data do not support this idea, as the slopes between the metabolic parameters and percent lesion coverage were similar between temperatures, such that the interaction term in the model was not significant (Fig. 4.1). It is possible that the AGD infection levels in this study may not have been high enough to uncover a detrimental impact of a temperature-induced decrease in aerobic scope, but such an idea must await future research utilising fish with AGD loads that are beyond industry treatment thresholds (i.e., >~20% lesion cover (Taylor et al., 2009a)). Notably, the data suggest that disease management thresholds could be similar across temperatures ranging from 15 to 19°C.

The values of $\dot{M}O_{2rest}$ in control fish are comparable with previous reports for salmonids (Brett and Glass, 1973; Henry and Houston, 1984; Evans, 1990). The significant linear increase in $\dot{M}O_{2rest}$ that occurred with increasing percent lesion coverage in both temperatures suggests an increase in basal metabolic requirements associated with disease progression. Fish with a history of heavy AGD have been observed to be smaller than their lightly infected conspecifics (Powell et al., 2002b), which could be at least partially explained by the rise in $\dot{M}O_{2rest}$ with AGD severity observed here. The combined observations of higher $\dot{M}O_{2rest}$ and lower growth rates suggest that energy typically directed towards growth may be diverted to fight the disease and maintain critical functions such as ionoregulation and acid-base regulation (Powell et al., 2005). However, the ion regulatory cost in the energy budget is a matter still under investigation (see review by Boeuf and Payan 2001). For example, studies have found 20 to >50% of the energy budget attributed to osmoregulation dependent on species (e.g. rainbow trout, catfishes [*Ictalurus punctatus* Rafinesque and *Ameiurus nebulosus* Lesueur], minnow [*Phoxinus erythrogaster* Rafinesque] and killifish [*Fundulus catenatus* Storer]) (Rao 1968; Nordlie and Lefler 1975; Nordlie 1978; Furspan et al. 1984; Nordlie et al. 1991; Toepfer and Barton 1992). On the other hand, Morgan and Iwama (1999) found an energy budget of <4% attributed to osmoregulation in cutthroat trout *(Oncorhynchus clarkii* Richardson) indicating results can vary based upon species and highlighting the need for further research into the area.

The finding of an increase in $\dot{M}O_{2rest}$ with percent lesion coverage supports results from previous studies (Powell et al., 2005; Leef et al., 2007c), but differs from studies that found no differences in $\dot{M}O_2$ between AGD-infected and uninfected individuals (Powell et al., 2000; Fisk et al., 2002; Powell et al., 2005; Leef et al., 2007c). Notably, however, methodological variations in AGD quantification can make direct comparisons with other studies difficult. For example, Powell et al. (2000) and Fisk et al. (2002) sourced all fish (adults) from a commercial farm during a freshwater bathing treatment; the former classified fish as either clear (no visible lesions) or heavy (established *Paramoeba* sp. lesions), while the latter classified fish as low (clear to very light infections) or high (light to heavy infection). Leef et al. (2007) and Powell et al. (2005), on the other hand, measured the experimental animals pre- and post-inoculation with amoebae. By grouping fish into AGD infected versus uninfected, it is not possible to understand the progression of effects from lighter- to heavier-infected fish within the infected group.

In contrast with $\dot{M}O_{2rest}$, $\dot{M}O_{2max}$ was unexpectedly independent of percent lesion coverage such that absolute and factorial aerobic scope declined as the AGD level progressed. The maintenance of $\dot{M}O_{2max}$ indicates that there was no effect of AGD on the maximum oxygen uptake capacity of the gills. While evidence suggests that reduced functional gill surface area reduces $\dot{M}O_{2max}$ (Duthie and Hughes, 1987; Davison et al., 1990), the findings are similar to the only other reported measurements of active $\dot{M}O_2$ in AGD-infected Atlantic salmon (Powell et al., 2005; Leef et al., 2007c). Thus, either AGD does not impact oxygen uptake capacity or, perhaps more likely, the level of infection in this and the previous studies was not high enough to reduce the functional gill surface area to the point where $\dot{M}O_{2max}$ is impacted. Furthermore, there were no differences in haematological parameters between infection statuses (Table 4.2), in similarity to previous work (Powell et al., 2000; Leef et al., 2007b), which suggests that the AGD infection did not induce hypoxaemia by limiting O_2 diffusion across the gill epithelium. Whether higher AGD loads cause a decrease in functional gill surface area and $\dot{M}O_{2max}$ remains to be tested, but the present study suggests that current AGD treatment thresholds in Atlantic salmon aquaculture are sufficient for preventing limitations to oxygen uptake.

Anaerobic recovery and hypoxia tolerance

Major physiological disturbances (e.g., oxygen stores, ion regulation, acid-base status) occur in fishes during exhaustive anaerobic exercise, and EPOC represents the oxygen/energy required to re-establish physiological homeostasis (Wood, 1991). The magnitude and duration of EPOC in this study were not influenced by the percent lesion coverage of the gills, and acclimation temperature only had an effect on the duration (Fig. 4.2). The latter coincides with previous studies that report a more rapid EPOC with increasing temperature (Kieffer and Tufts, 1996; Galloway and Kieffer, 2003). Thus, this EPOC data provide evidence that the re-establishment of physiological homeostasis following exercise is not compromised by AGD at levels typically found in aquaculture.

Conversely, some evidence was found that hypoxia tolerance may have been at least partly compromised at the higher AGD levels. Indeed, while both P_{crit} and DO at LOE increased significantly with temperature, DO at LOE also increased with percent lesion coverage (Fig. 4.3). The increase in P_{crit} and DO at LOE with temperature is consistent with most previous studies of other species, and it results from a temperature-dependent increase in oxygen demand (i.e., $\dot{M}O_{2rest}$) causing a decrease in hypoxia tolerance (Ott et al., 1980; Fernandes

and Rantin, 1989; Schurmann and Steffensen, 1997; Collins et al., 2013b). The increase in DO at LOE with percent lesion coverage may result from a similar mechanism, whereby the elevated $\dot{M}O_{2rest}$ of highly infected fish causes a reduction in hypoxia tolerance. While the trend for an increase in P_{crit} with percent lesion coverage lends further support to this idea (Fig. 4.3), the regression models did not reach statistical significance.

The authors are not aware of any previous study that has explicitly tested the impacts of AGD on hypoxia tolerance, although a couple of related studies exist. In a graded hypoxia trial down to ~25% air saturation, it was reported that the presence of AGD did not contribute to respiratory failure beyond that observed in control fish, despite signs of respiratory acidosis in infected fish (Powell et al., 2000). In another study, resting $\dot{M}O_2$ was significantly lower when measured in hypoxia (50% air saturation) versus normoxia in high AGD-infected fish but not in fish exhibiting low AGD loads (Fisk et al., 2002). Thus, existing evidence suggests that AGD may cause some reduction in hypoxia tolerance, which may become exacerbated as the disease progresses and gill lesion coverage increases.

Conclusions

While AGD proliferates at high temperatures in aquaculture, and temperature alone causes an increase in metabolic rate and a decrease in hypoxia tolerance, no interaction was found between AGD and temperature for the variables measured. That is, the effects of AGD on $\dot{M}O_2$, EPOC and hypoxia tolerance are independent of temperature under the conditions examined here. However, within both temperatures, the percent lesion coverage of the disease on the gills increased $\dot{M}O_{2rest}$, decreased absolute and factorial aerobic scope, and increased DO at LOE. Therefore, even within the AGD loads accepted by the Atlantic salmon aquaculture industry (<20% lesion coverage), there is evidence of whole-animal performance reductions. It is possible that the increase in $\dot{M}O_{2rest}$ and decrease in absolute and factorial aerobic scope may contribute to decreased growth and increased lethargy commonly observed in infected fish (Rodger and McArdle, 1996), and there is a need to understand how fish size may interact with the physiology and environmental tolerance of AGD-infected fish. While existing management of AGD in salmon aquaculture seems appropriate to avoid major performance decrements, the data suggest that even low levels of AGD may cause sub-lethal impairments and may even exacerbate mortality during hypoxic periods.

Chapter 5: General discussion

This thesis aimed to elucidate the potential impacts of global warming and summer heatwaves on the performance of aquaculture-reared Atlantic salmon, with particular emphasis on the interactive effects of AGD. There were negligible differences in metabolism or thermal tolerance between diploid and triploid Atlantic salmon (Chapter 2). However, AGD lowered acute thermal tolerance of diploid Atlantic salmon at high AGD infection levels (Chapter 3). While acclimation to 19°C elevated metabolism above that of 15°C-acclimated salmon, there was no temperature and disease interaction, indicating that fish at the warmer temperature performed equally as well as those at the cooler temperature for a given AGD level. Within each temperature, increasing AGD infection resulted in an increase in basal metabolic costs ($\dot{M}O_{2rest}$) and a decrease in aerobic scope (Chapter 4). Furthermore, hypoxia tolerance was impaired in AGD-infected salmon, as evidenced by an increase in DO at LOE (Chapter 4). These findings are expanded on below, particularly in the context of how AGD is likely to impact the ability of Atlantic salmon to cope with environmental disturbances now and into the future.

Ploidy effects on salmon physiology

Contrasting results exist regarding physiological differences between diploid and triploid Atlantic salmon. In Chapter 2, significant differences were found in mass, SGR, and $\dot{M}O_{2rest}$ at the beginning of the study, but those differences disappeared by week 7 (Figs. **2.2**, **2.3**). It is possible that these results are a product of behavioural differences between diploids and triploids being held in communal tanks. Indeed, diploids are postulated to be more aggressive than triploids, contributing to diploids out-competing triploids for food when held together (Carter et al., 1994; Galbreath et al., 1994). Moreover, standard metabolic rate has been correlated with dominance hierarchies in intraspecific populations (Metcalfe et al., 1995; Cutts et al., 1998; Norin et al., 2016), which may be relevant to the higher $\dot{M}O_{2rest}$ and SGR exhibited by diploids. Furthermore, body size is known to be negatively associated with growth rates (Iwama, 1996b) and diploids were significantly smaller at week 0. These factors could have been acting in tandem to drive the higher growth rates of diploids at the beginning of the experiment.

Despite published observations of lowered performance and metabolic rate of triploid fish at high water temperatures (Ojolick et al., 1995; Altimiras et al., 2002; Hyndman et al., 2003; Atkins and Benfey, 2008), negligible differences were found in thermal tolerance or

metabolism between ploidies. Triploid Atlantic salmon have been reported to have a higher routine metabolic rate at lower temperatures (12°C) and a lower routine metabolic rate at higher temperatures (18°C), which the authors suggested is indicative of a lower thermal optima in triploids compared to diploids (Atkins and Benfey, 2008). This contrasts with the findings of lower resting metabolism in triploids than diploids at all acclimation temperatures and during the CT_{max} protocol. Despite the lower routine metabolic rate of triploids during this experiment, no evidence was found of lowered thermal tolerance in triploids compared to diploids, suggesting that aerobic metabolism and acute thermal tolerance are not causally related.

It is important to note that the ploidy experiments in Chapter 2 were conducted in freshwater, so it is worthwhile considering how salinity may differentially influence the physiological performance of diploids and triploids. Once Atlantic salmon, being an anadromous species, transitions to seawater, issues associated with ionic regulation are reversed. In freshwater, the environment is hypo-osmotic and salmon are subject to osmotic flooding and losing ions to the surrounding water, whereas the opposite is true in the hyperosmotic marine environment (Hoar, 1988). While whole animal oxygen consumption rates increase with seawater acclimation in coho salmon (Morgan and Iwama, 1998), it is difficult to determine what proportion of the total oxygen consumption can be attributed to ion regulation at the site of the gill (Hwang et al., 2011). Efforts to elucidate this have used isolated gill perfusions and found that <20% of total oxygen consumption was attributed to gill tissue in Atlantic salmon (McCormick et al., 1989) suggesting that ion regulation on seawater transfer may have a relatively small impact on the increase in oxygen consumption seen at the whole-animal level. The effects of the larger cell size of triploids on ion regulation are not known (Shrimpton et al., 2012), but Sadler et al. (2001) showed that triploid Atlantic salmon have a significantly smaller gill surface area than diploids. With a smaller surface area, it is possible that triploid Na^+, K^+ ATPase activity may be elevated to maintain the necessary ionic homeostasis (Shrimpton et al., 2012). After a 24 h seawater challenge, Shrimpton et al. (2012) found lower Na^+, K^+ ATPase activity in triploids compared to diploids, but sampling following a four month seawater grow-out period showed the reverse, elevated Na^+, K^+ ATPase activity in triploids compared to diploids. This delay in increased ATPase activity could play some role in the lower smolting rates observed in triploid coho salmon (Withler et al., 1995) and Atlantic salmon (O'Flynn, 1997).

One of the consequences of triploidy induction is fewer but larger cells compared with diploid counterparts. Indeed, erythrocyte counts have been observed to be lower in triploids while haematocrit has been maintained (Sadler et al., 2000). In addition, the larger cell size reduces the surface area-to-volume ratio, potentially having consequences for cell transport processes in triploids (Maxime, 2008). Oxygen uptake at the gills is a diffusive process (Dejours, 1981) indicating that the increased erythrocyte size could have implications for haemoglobin loading during periods of stress. However, similar performance between diploids and triploids was observed under the acute thermal challenge (Chapter 2), suggesting that cell size and potential diffusion issues did not impair thermal tolerance. Similarly, triploid Atlantic salmon have been shown to maintain respiratory homeostasis the same as diploids when challenged with 2.5 h of confinement stress (Sadler et al., 2000). Furthermore, Chinook salmon triploids performed similarly in a sustained exercise challenge, although triploids showed a smaller oxygen carrying capacity than diploids, so the authors suggested that triploids could compensate different parameters of oxygen transport to maintain performance under the exercise regime utilized (Bernier et al., 2004). While the exact mechanisms underlying compensation were not investigated (Bernier et al., 2004), fish can compensate for an exercise-induced reduction in O_2 carrying capacity through increasing arterial-venous O_2 extraction, cardiac output, ventilation volume and frequency (Kiceniuk and Jones, 1977), by shunting blood from the gut towards aerobic red muscle (Thorarensen et al., 1993), or by recruiting anaerobic white muscle fibres and repaying the oxygen debt post-exercise (Jones, 1982). While the mechanism of compensation should be empirically investigated, these results suggest that triploids have the capacity to compensate for their larger but fewer cells. Nevertheless, the results do not help to explain the observed susceptibility of triploids to suboptimal environments, and thus this remains a fruitful avenue for future research (Johnson et al., 1986; Quillet and Gaignon, 1990; Jungalwalla, 1991; Yamamoto and Iida, 1994; Ojolick et al., 1995).

While ploidy did not affect the relative ventricle mass in this thesis (Table 2.1), there is some evidence for altered heart morphology in triploid Atlantic salmon. Triploid ventricles have been reported to be more triangular than diploids (Leclercq et al., 2011), therefore bearing more resemblance to the wild-type rather than domesticated salmonids (Poppe et al., 2003). The alteration in morphology has been suggested to improve ventricular contraction and thereby oxygen delivery, which otherwise has been proposed to be impaired in triploids

(Bernier et al., 2004; Leclercq et al., 2011; Verhille et al., 2013). Nevertheless, reports are inconsistent (Fraser et al., 2012), and triploid ventricles have been reported to be rounder than diploid ventricles in a more recent study (Fraser et al., 2014). Given that there is a strong correlation between heart morphology and function, the altered heart morphology of triploids in some studies may impact performance compared with diploids (Graham and Farrell, 1992; Agnisola and Tota, 1994; Sanchez-Quintana et al., 1995; Sande and Poppe, 1995; Coucelo et al., 1996; Tota and Gattuso, 1996). For example, a more triangular heart has been linked to improved swimming and cardiac performance in rainbow trout (*Oncorhynchus mykiss* Walbaum) (Claireaux et al., 2005). While heart shape was not investigated in Verhille et al. (2013), ventricle mass was similar between rainbow trout diploids and triploids. Despite this, cardiac arrhythmia occurred in 100% of triploids at a temperature (22°C) where 30% of diploids still maintained rhythmic heartbeats (Verhille et al., 2013). Therefore, triploid poor performance at high temperatures could be linked to cardiovascular dysfunction rather than ventricle size, but further research is necessary to thoroughly elucidate the mechanisms underlying differential thermal performance across ploidies.

AGD effects on salmon physiology

Increasing AGD infection increased the basal metabolic needs of Atlantic salmon smolts at acclimation temperatures of both 15 and 19°C (Chapter 4). Basal energy requirements are governed by a great range of factors including ambient temperature, osmo- and ion-regulation, digestive state, and immune responses (Brett, 1964; Rao, 1968; Beamish, 1974; Morgan and Iwama, 1991; Houston et al., 2007). Rainbow trout, a salmonid, has been shown to only perfuse ~58% of the gill surface area at rest with the ability to recruit lamellae during periods of exercise to effectively increase the functional gill surface area for oxygen uptake (Booth, 1978). Indeed, a later study that experimentally reduced gill surface area of rainbow trout by 30% through cauterization of filaments reported no difference in $\dot{M}O_{2rest}$, suggesting a recruitment of lamellae to defend respiration (Duthie and Hughes, 1987). The relatively low level of gill damage (<10% lesion coverage) seen in Chapter 4 suggests that the increase in basal $\dot{M}O_2$ is not a result of the reduced surface area, but rather a product of the increased energy required to cope with the disease. Specifically, immune responses in animals can be energetically costly (Houston et al., 2007), and therefore likely contributed to the observed increase in $\dot{M}O_2$ with increasing AGD infection levels.

In this context, an up-regulation of the pro-inflammatory cytokine IL-1β has been observed in association with lesions in AGD-infected fish (Bridle et al., 2006b; Morrison et al., 2006; Pennacchi et al., 2014; Pennacchi et al., 2016). Excess mucus is commonly observed in association with AGD lesions and may be the result of IL-1β (Bridle et al., 2006a) which is known to increase and alter mucus in various mammalian epithelial cells (Takahashi et al., 1998; Enss et al., 2000)(Takahashi et al.). Furthermore, mucus cell populations have been observed to proliferate in the gills of AGD-infected individuals (Roberts and Powell, 2003a). Mucus has been proposed to influence processes such as respiration, ionic and osmotic regulation, defence against disease and environmental perturbations (Shephard, 1994). While gill mucus production does not appear to affect oxygen uptake, there is evidence that carbon dioxide excretion becomes impaired (Powell and Perry, 1996; Powell and Perry, 1997; Powell and Perry, 1999). This could underlie the characteristic respiratory acidosis observed in AGD-infected Atlantic salmon (Powell et al., 2000; Powell and Nowak, 2003). In addition, mucus viscosity can play a role in the permeability of ions across the gill (Shephard, 1994). Mucus is a polyanionic gel (Verdugo, 1984), indicating that anions would have a greater diffusive potential compared to cations, and the latter could become bound within the mucus layer (Zuchelkowski et al., 1985). The cutaneous mucus layer of AGD-infected Atlantic salmon has been reported to be less viscous (Roberts and Powell, 2005) with more extensive secretion (Munday et al., 1990; Nowak and Munday, 1994; Adams and Nowak, 2003; Roberts and Powell, 2003a) than that of their naive counterparts. Munday et al. (2001) observed that in the later stages of AGD development, hyperplastic gill tissue and lesion-associated amoebae tended to slough off. The thinner mucus layer of AGD-infected fish could potentially aid this 'self-cleaning' action (Roberts and Powell, 2003b). With no significant change in numbers of chloride cells with AGD infection (Powell et al., 2001), the alterations to the mucus layers (both cutaneous and branchial) could have effects on ionic and osmotic regulation and could potentially result in the elevation of $\dot{M}O_{2rest}$ in order to maintain homeostasis.

A further indication that the gills remained uncompromised at aquaculture-relevant infection levels is evidenced by the similar $\dot{M}O_{2max}$ values achieved by infected and uninfected fish in Chapter 4 (Fig. 4.1). If lesion coverage was high enough to decrease the functional gill surface area, then there theoretically should have been a decrease in $\dot{M}O_{2max}$. Indeed, in the rainbow trout study mentioned above, Duthie et al. (1987) observed a decrease in $\dot{M}O_{2max}$ with the 30% reduction in gill surface area. Similarly, bald notothen

(*Pagothenia borchgrevinki*, Boulenger) infected with X-cell gill disease experienced a reduction in $\dot{M}O_{2max}$ with percent gill damage due to the disease (Davison et al., 1990). On the other hand, similar to this thesis, a necrotic bacterial gill infection of Atlantic salmon resulted in increased $\dot{M}O_{2rest}$ but similar $\dot{M}O_{2max}$ (Jones et al., 2007). While the latter study did not measure the amount of gill damage hindering oxygen uptake, the former two (Duthie and Hughes, 1987; Davison et al., 1990) reported gill damage greater than in this thesis (30% and >20%, respectively).

In a recent study, AGD-infected Atlantic salmon exhibited compromised aerobic scope, similar to the findings in this thesis, however it was through similar $\dot{M}O_{2rest}$ values but lower $\dot{M}O_{2max}$ values compared to uninfected fish measured in group-based respirometry (Hvas et al., 2017). While infection levels reached greater amoebae loads in Hvas et al. (2017), methodological variations could also help explain the differences between the studies. In this thesis, overnight intermittent flow respirometry and a chase protocol were used for $\dot{M}O_{2rest}$ and $\dot{M}O_{2max}$, respectively. Hvas et al. (2017) utilized a critical swimming protocol that accepts the highest $\dot{M}O_2$ value as the maximum and extrapolates swimming velocity down to 0 cm s^{-1} to calculate $\dot{M}O_{2rest}$ based upon Brett (1964). However, fish may show restless behaviour, especially at low swimming speeds, so extrapolation may overestimate $\dot{M}O_{2rest}$ values (Brett, 1964). On the other hand, some studies have demonstrated that the extrapolation method and resting respirometry concur in $\dot{M}O_2$ estimations (Schurmann and Steffensen, 1997; Roche et al., 2013). Despite methodological differences, both studies concur that AGD can influence metabolism and reduce aerobic scope.

In addition to oxygen uptake capacity at the gills, haematological parameters are also important in determining the respiratory capacity of organisms. Haemoglobin, haematocrit, and MCHC were found to be similar between controls and AGD-infected fish under both acute (Chapter 3) and chronic (Chapter 4) thermal regimes. Haemoglobin remained stable under acute and chronic thermal challenges, while haematocrit and MCHC remained stable under chronic conditions but increased and decreased, respectively, under acute thermal increases (Table 4.2 and Fig. **3.3**). The decrease in MCHC under the acute thermal challenge is indicative of red blood cell swelling associated with a stress response (Gallaugher and Farrell, 1998). Similarly to this thesis, Leef et al. (2005) did not find any differences between AGD-infected and control fish in haematological parameters over 96 h after inoculation. Haematocrit and haemoglobin directly influence the oxygen carrying capacity of the blood (Gallaugher and Farrell, 1998). Haematocrit, in particular, is a direct determinant of arterial

oxygen content (Gallaugher and Farrell, 1998). An elevation in haematocrit, therefore, could help mitigate any potential oxygen uptake limitations at the gill associated with AGD (Leef et al., 2005a). The similarities in haematocrit between control and AGD-infected fish under chronic and acute thermal conditions lend support to the idea that under AGD levels seen in this thesis, oxygen uptake capacity was not limited.

To the author's knowledge, EPOC has only been measured once in AGD-infected Atlantic salmon (reported as 'unpublished data') and was reported to be greater in severely infected fish (Powell et al., 2008). The objective during recovery from exhaustive exercise is to restore physiological homeostasis with as little metabolic cost as possible (Wood, 1991). As in mammals, it is possible to separate EPOC into two components: a 'fast' component and a 'slow' component. Scarabello et al. (1991) found that the fast component contributed about one-fifth to the total EPOC while the slow component accounted for the remaining four-fifths in juvenile rainbow trout after a three minute chase protocol. Within the fast component, 83% could be explained through restoration of creatine phosphate and ATP and oxygen stores, while only 25% of the slow component could be explained through lactate clearance and glycogen resynthesis (Scarabello et al., 1991). If AGD-infected gills had an oxygen uptake limitation, then it may be expected that total EPOC would be of longer duration, as reported previously as 'unpublished data' (Powell et al., 2008). However, no differences were seen in magnitude or duration of EPOC with increasing AGD levels. This is unsurprising as the authors did not observe any differences in $\dot{M}O_{2max}$. Therefore, the peaks of the EPOC curves were similar and as suggested previously, the percentage of affected gill area in this study likely did not hinder oxygen uptake to replenish stores and return to physiological homeostasis.

Cardiac failure has also been proposed as a possible cause for AGD related mortality in salmon (Leef et al., 2007c). While no significant differences were found in relative ventricular mass with AGD infection, some studies have found cardiac dysfunction with AGD that could contribute to death. Cardiac output has been reported to be compromised in AGD-infected Atlantic salmon (Powell et al., 2005; Leef et al., 2005a). Interestingly, dorsal aortic pressure was slightly elevated in AGD-infected individuals in both studies, albeit not significantly (Powell et al., 2005; Leef et al., 2005a), which contrasts with an earlier study which observed significantly higher dorsal aortic pressure in unbathed AGD-infected Atlantic salmon compared with bathed control fish (Powell et al., 2002a). Heart rate, however, remained stable so the higher dorsal aortic pressure was suggested to be a result of

increased systemic vascular resistance (Powell et al., 2002a), which was confirmed in later studies (Powell et al., 2005; Leef et al., 2005a). Leef et al. (2007) tested all of these parameters in one study and observed the same trends of decreased cardiac output and increased systemic vascular resistance of AGD-infected individuals compared with controls. The authors also observed a decreased stroke volume which contributed to the lowered cardiac output since heart rate once again remained stable. The most severely infected individuals exhibited elevated dorsal aortic pressure resulting in hypertension which was not seen in moderately infected or control individuals. Therefore, the individuals in the study conducted by Powell et al. (2002) could have been more severely infected than those used in later studies. It indicates that Atlantic salmon has the ability to defend dorsal aortic pressure through changes to other cardiovascular parameters to a certain extent (Leef et al., 2005a; Leef et al., 2007b). Furthermore, fish with a history of heavy AGD exhibited altered morphometrics of the heart. The ratios of ventricle axis length and width and axis length and height were significantly higher and there was an overall thickening of the muscularis compactum in the hearts of fish with a heavy AGD history (Powell et al., 2002b). While there is capacity for great morphological plasticity of the heart within a species (Poppe et al., 2003), any deviation from the pyramidal (triangular) shape, which is important for optimal cardiac functioning (Poppe et al., 2002), could predispose individuals to cardiac failure during periods of stress (Powell et al., 2008). Combined, these results suggest that cardiac function could become compromised under stressful conditions, such as a CT_{max} protocol, and therefore contribute to AGD-associated death (Powell et al., 2002a; Powell et al., 2002b; Powell et al., 2005; Leef et al., 2005a; Leef et al., 2007b).

Cardiovascular parameters including relative ventricle mass (RVM) have also been found to be correlated with CT_{max} (Anttila et al., 2013). Fish with a larger RVM have been found to have a higher stroke volume (Franklin and Davie, 1992), which could translate to a higher cardiac output as it is the product of stroke volume and heart rate. A higher cardiac output could improve oxygen supply to the tissues during periods of high temperature and translate into a higher CT_{max} (Anttila et al., 2013). However, this is not always the case. Cold-acclimated salmonids consistently exhibit a larger RVM but their upper thermal tolerance is reduced (Farrell et al., 1988; Klaiman et al., 2011), highlighting the complex interplay between cardiac mass, thermal acclimation and acute thermal tolerance. Moreover, there is growing evidence that CT_{max} is not driven by oxygen limitation until severe ambient hypoxia is reached (Brijs et al., 2015; Ern et al., 2016). No differences in RVM between infected and

uninfected individuals were observed during acute or chronic thermal exposures, so this is unlikely to have played a role in the reduced upper thermal tolerance of severely infected individuals in Chapter 3.

AGD effects on performance in aquaculture

The magnitude of aerobic scope is an indication of the capacity of individuals to elevate oxygen uptake above baseline energy requirements to fuel key life functions such as digestion, locomotion, growth, and reproduction (Guderley and Pörtner, 2010). Somatic growth, in particular, is a valuable trait in the aquaculture industry which could be compromised by a reduction in aerobic scope with increasing AGD. Indeed, observations from Atlantic salmon farms in Chile saw a reduced feed intake and a reduction in growth rate of up to 25% in infected fish (Bustos et al., 2011). Furthermore, the observed reduction in aerobic scope in Chapter 4 could help explain observations of lethargy and apparent respiratory distress of infected fish (Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996).

Aerobic scope decreased with increasing AGD (Chapter 4, Fig. 4.1), and upper acute thermal tolerance, as measured by CT_{max}, was reduced in Atlantic salmon exhibiting high AGD levels (gill scores 4 and 5) (Chapter 3, Fig. **3.2**). While aerobic scope and CT_{max} may seem correlated, there is strong evidence to suggest that the lowered CT_{max} at high AGD levels (Chapter 3) is not caused by the reduction in aerobic scope (e.g., Brijs et al. (2015)). While the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (Pörtner and Knust, 2007; Pörtner and Farrell, 2008) has been widely publicised, it has also received significant debate (Clark et al., 2013; Norin et al., 2014; Schulte, 2015; Lefevre, 2016). The hypothesis postulates that aerobic scope universally governs performance and fitness of aquatic ectotherms. However, in European perch (*Perca fluviatilis* Linneaus) CT_{max} was unchanged with a doubling of aerobic scope or about a 43% reduction in haemoglobin concentration (induced by hyperoxia and anemia, respectively) (Brijs et al., 2015). Furthermore, juvenile barramundi (*Lates calcarifer*) continuously increased aerobic scope to 38°C during an acute temperature exposure, which is close to their thermal maximum of 41°C, but when acclimated to 38°C, aerobic scope decreased to similar values as for 29°C-acclimated fish (Norin et al., 2014). Therefore, the authors suggest that the decrease seen in aerobic scope with increasing AGD is unlikely to be the causal factor underlying the decline in CT_{max} at high infection levels. In any event, it is likely that severely infected Atlantic salmon will be compromised during heatwaves because of their lower CT_{max}. Notably, however, the

Tasmanian Atlantic salmon industry typically initiates freshwater bathing practices when the stock reaches an average gill score of ~3. Based on the data presented here, this seems like an appropriate AGD cut-off score to prevent the stock from having compromised acute thermal tolerance.

Aside from the effects of AGD on acute thermal tolerance, chronically elevated temperature is known to be a determinant environmental factor in AGD progression (Munday et al., 2001). Interestingly, however, there was no difference in disease progression between fish acclimated to 15 or 19°C, as quantified using percent lesion coverage (Chapter 4). Potential factors precluding increased disease progression at 19°C compared to 15°C could be due to stocking density or the virulence of the strain of *P. perurans*. Furthermore, it was expected that fish held at 19°C would not cope as well with the disease, but this was not evident since the regression lines for all measured metrics (as a function of percent gill lesion coverage) had similar slopes across temperatures (Figs. 4.1, 4.2, 4.3). In other words, the performance impacts of the disease appeared consistent across the two acclimation temperatures, suggesting that there may be scope for acclimation with progressive increases in average seasonal temperatures.

Hypoxia tolerance and capacity for anaerobic exercise in AGD-infected fish have received little attention to date. Evidence for impaired hypoxia tolerance was found as the percent of affected gill area increased (Chapter 4). Indeed, a previous study reported reduced survival of AGD-infected fish after a hypoxic challenge of 50% air saturation compared to controls (21.4% versus 88.9% survival) (Fisk et al., 2002). Furthermore, $\dot{M}O_2$ was reported to be significantly reduced in the severely infected group from normoxia to hypoxia, while no such reduction was found in the controls (Fisk et al., 2002). Whether the reduction in $\dot{M}O_2$ contributed to the poorer survival of infected fish at 50% air saturation remains unknown in the absence of research investigating the causal factor(s) responsible for hypoxia-induced mortality associated with AGD. Despite the absence of an identified mechanism, it seems likely that the current freshwater bathing regime in the aquaculture industry (bathing initiated once average gill score is ~3) is sufficient to prevent significant impairments in hypoxia tolerance and recovery from anaerobic exercise.

Conclusions and future directions

This thesis has shown that triploid and diploid thermal physiology is similar during the freshwater stage of the life cycle, and that sub-optimal environments negatively impact AGD

infected individuals compared with uninfected controls. Acute thermal tolerance and hypoxia tolerance are impaired as AGD infection progresses. Furthermore, AGD-infected individuals exhibit a compromised aerobic scope, which may be a contributing factor to the commonly observed lethargy seen in infected stocks.

Further investigations should be undertaken to conclusively determine the benefits and drawbacks of triploids versus diploids in aquaculture. While this thesis shows that triploids perform similarly to diploids in freshwater, this represents only a modest portion of the Atlantic salmon lifecycle. In particular, determining the environmental tolerance of triploids during the marine grow-out phase is paramount in understanding their suitability to be farmed into the future, with the projected increases in average temperatures, heatwaves, and periods of hypoxia. Furthermore, in the marine environment, the fish will be faced with diseases and challenges not seen in the freshwater hatchery (e.g. AGD and ionic and osmotic regulation in saltwater). The inherent sterility of triploids is useful for aquaculture as it allows triploids to reach market size without the stress of maturation. Their sterility also has drawbacks as they cannot be selectively bred for environmental tolerance. Therefore, it may be worth investigating family differences in thermal and hypoxia tolerance of diploids that could then be used to potentially form more tolerant triploids. While this is an interesting idea, little is known of whether environmental tolerance characteristics of diploid parents are retained when offspring are converted to triploids. In this context, the effect of ploidy should be investigated in relation to processes like ion regulation under sub-optimal environmental conditions. Ultimately, performance of triploids in forecasted aquaculture conditions will determine their suitability as a viable alternative to diploids as the salmon industry continues to evolve.

AGD is the biggest health issue facing salmon farms in Tasmania, which may be exacerbated with warming waters as the disease proliferates with temperature above ~15°C. The long-term goal of the Atlantic salmon aquaculture industry is, of course, to eradicate the disease altogether. However, in the shorter term, the Salmon Breeding Program aims to lengthen the time between freshwater baths and lessen the impact of AGD on the stock. This thesis shows that AGD does impair thermal and hypoxia tolerance as AGD increases. With Tasmania being a global warming 'hotspot' and increasing in temperature quicker than the global average (Ridgway, 2007; Frusher et al., 2013), thermal tolerance could be a valuable trait to select for in the breeding program. Knowledge of environmental tolerance is increasingly important for the stocks as the farms are interested in expanding to new

locations and potentially off-shore, bringing novel environmental challenges that may interact with performance and resilience to AGD.

One of the next steps from this thesis would involve the use of bio-loggers or telemetry in a field setting (i.e. aquaculture cages). Similar to the work done by Stehfest et al. (2017), bio-loggers could be implanted in salmon to quantify the DO, temperature, or activity profiles of infected and uninfected individuals. The data could provid insight into welfare, behaviour and/or mortality during environmental fluctuations and during routine farm practices such as freshwater bathing, gill scoring, or feeding. In addition, rapidly-evolving technologies in genomics and bioinformatics could be used to determine the key molecular processes underlying environmental resilience, AGD susceptibility and subsequent performance decrements, which may help to target the physiological processes to be assessed in future experimental investigations. Indeed, an understanding of the breadth and causes of genotypic and phenotypic diversity in environmental tolerance and AGD resilience would help to inform the existing salmon breeding program in Tasmania. Different salmonid species exhibit variable susceptibilities to AGD (Munday et al., 2001), providing the opportunity for novel comparative approaches to help pinpoint the underlying mechanisms of susceptibility. Ultimately, it is evident that multi-disciplinary research programs including fields such as physiology, genetics and immunology will yield the most fruitful outcomes for future-proofing the aquaculture industry in a changing world.

Appendix A: ImageJ analysis

Entire gill baskets were extracted and fixed in Davidson's seawater fixative. After 24 h, the gills were transferred to 70% ethanol. Arches were separated and photos taken of all 16 hemibranchs (4 per photo). Gill image analyses were conducted using ImageJ (details) and macros were written to streamline the analysis process.

Crop the images

First, the photos needed to be divided into four (one arch per photo) to reduce the size of the photo file. To that end, the first macro tells the user to choose two directories, one where the original photos are and the second where the cropped photos should be saved (Fig. A.1 and Fig. A.2, respectively). The next couple of lines of the macro create a loop through the original file directory opening one photo at a time (Fig. A.3). After a photo is opened, a rectangle is created for the next steps of the macro and a 'Wait for user' command is called with a prompt on what action the user should take. The first case is to position the rectangle over the top left gill arch and then press okay (Fig. A.4). The program then makes a duplicate of the area that was inside the rectangle, renames the duplicate, and saves it to the specified cropped photo directory (dir2), and finally closes the duplicated file (Fig. A.5). This is repeated for three more times to duplicate, crop, and save the top right and bottom left and right gill arches as well (Fig. A.6). The last command is 'Close All' which closes all open images. The macro then loops back to the start to open the next image in the original image directory (dir).

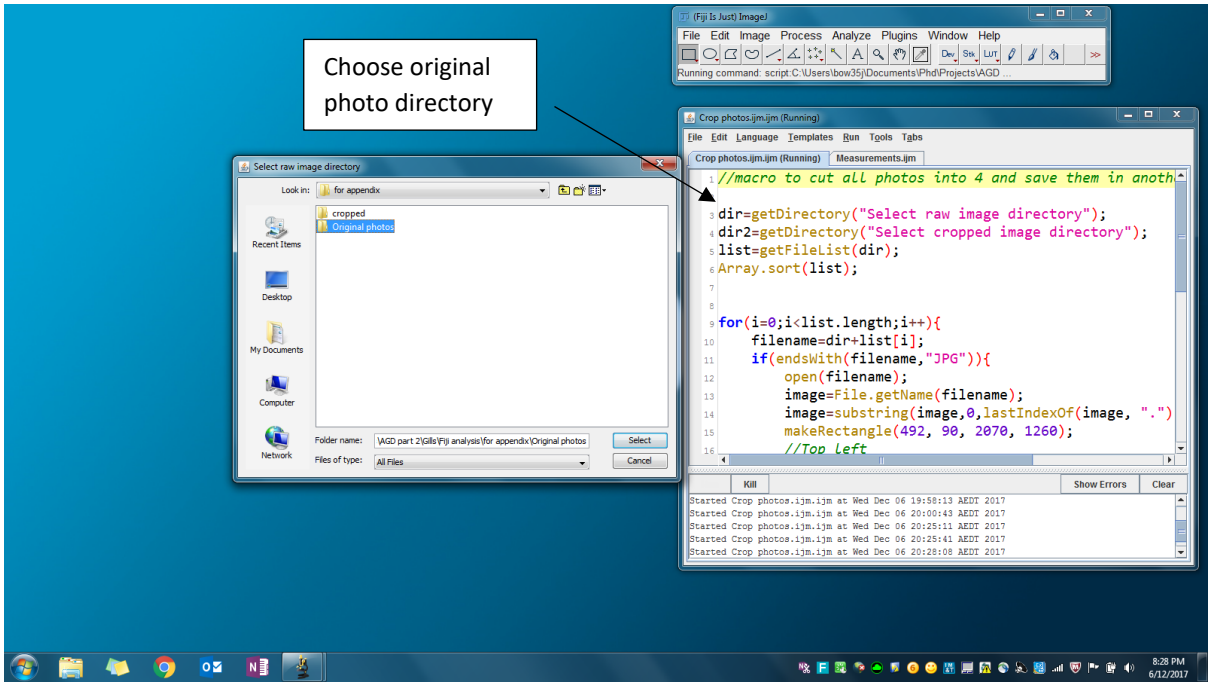

Figure A.1: First line of code opens Windows Explorer and prompts user to choose the directory containing the original images (dir).

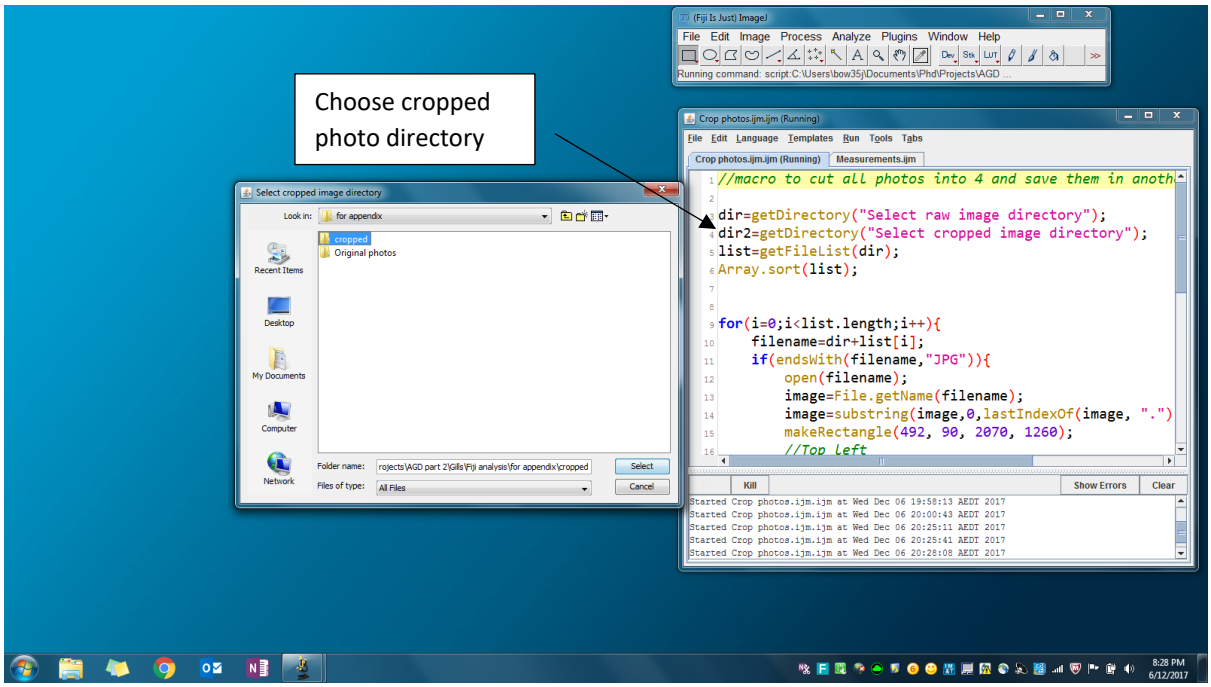

Figure A.2: The second line of the macro prompts the user to choose the directory where the cropped photos should be saved (dir2).

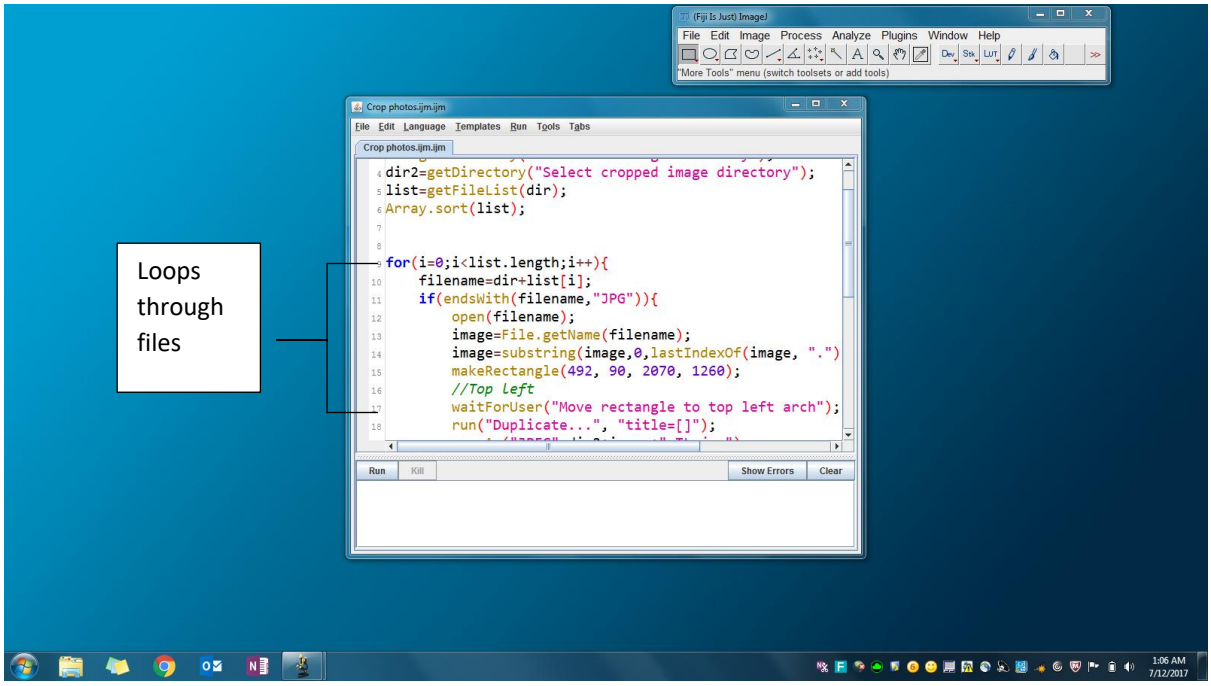

Figure A.3: Seven lines that open one file at a time and create a rectangle to crop the photo in the next steps of the macro.

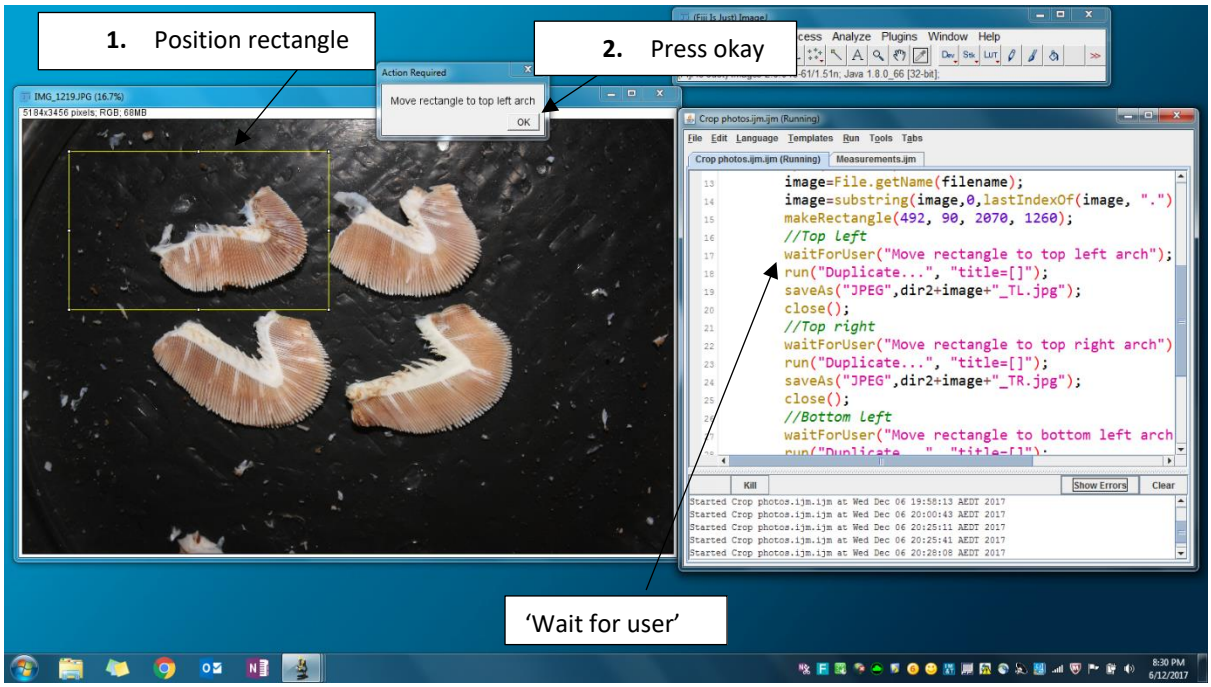

Figure A.4: The first 'Wait for user' command prompts the user to position the rectangle over the top left gill arch.

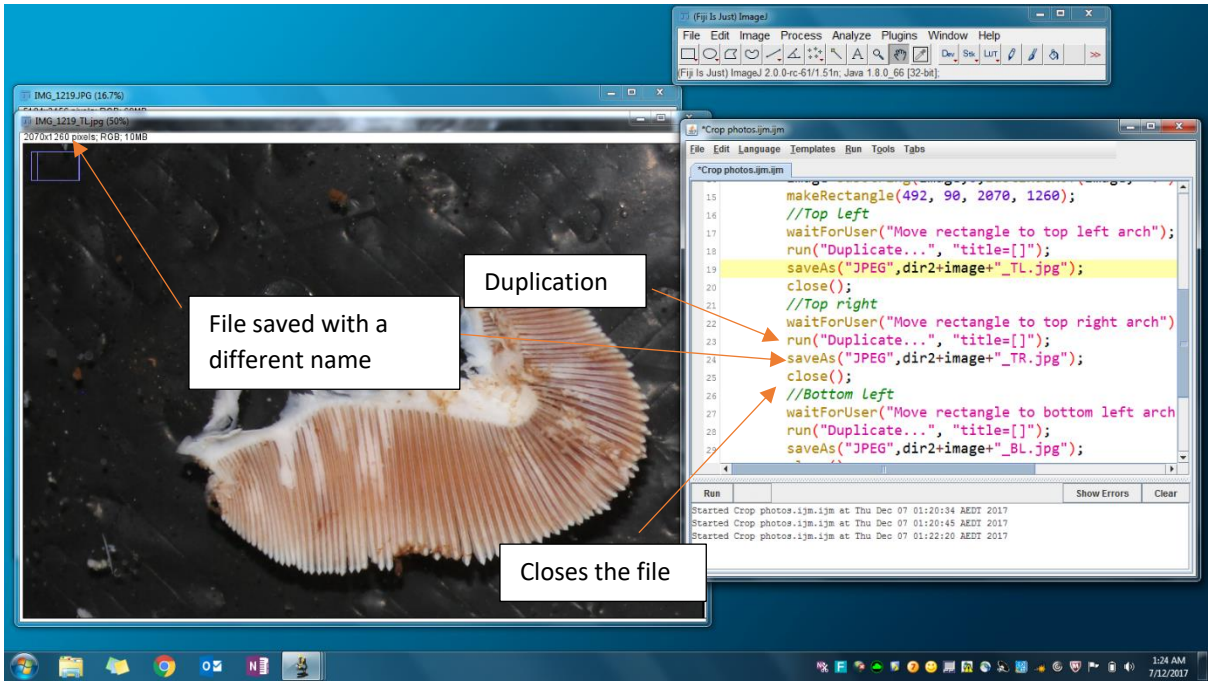

Figure A.5: The area in the rectangle is duplicated, the cropped picture saved with a new name and then the cropped photo closed.

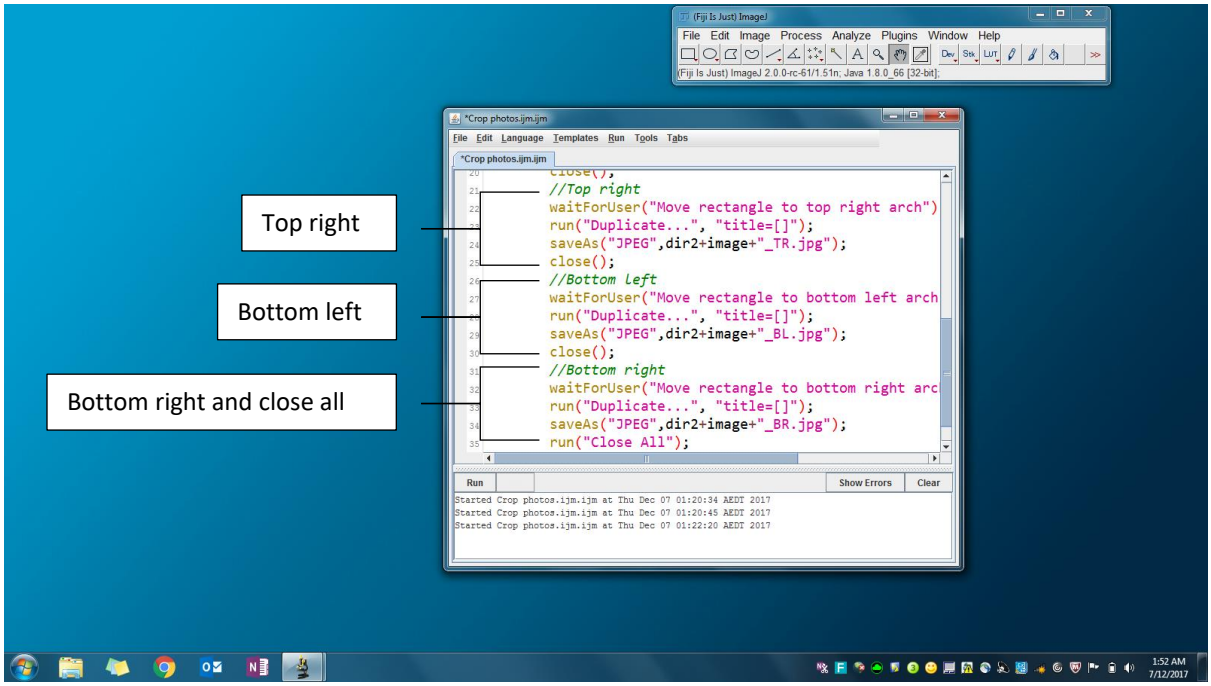

Figure A.6: Repeating the duplicating and saving steps for the top right and bottom left and right gill arches. The commands are followed by 'Close All' to close any open images.

Measurements

A second macro was written to conduct measurements in three steps: the whole gill, the arch, and the lesions. Similar to the macro above, the first couple of lines of code prompt the user to choose the cropped directory from above and then the mask directory for the files that will be saved (Figs. A.7 and A.8). The next section of the macro creates custom data tables to which the measurements are written (Fig. A.9). The next few lines are similar to above: a loop is created to open the first figure of the directory and loop through each file. Then the open image is split into three 8-bit images on the green, red, and blue RGB channels (Fig. A.10).

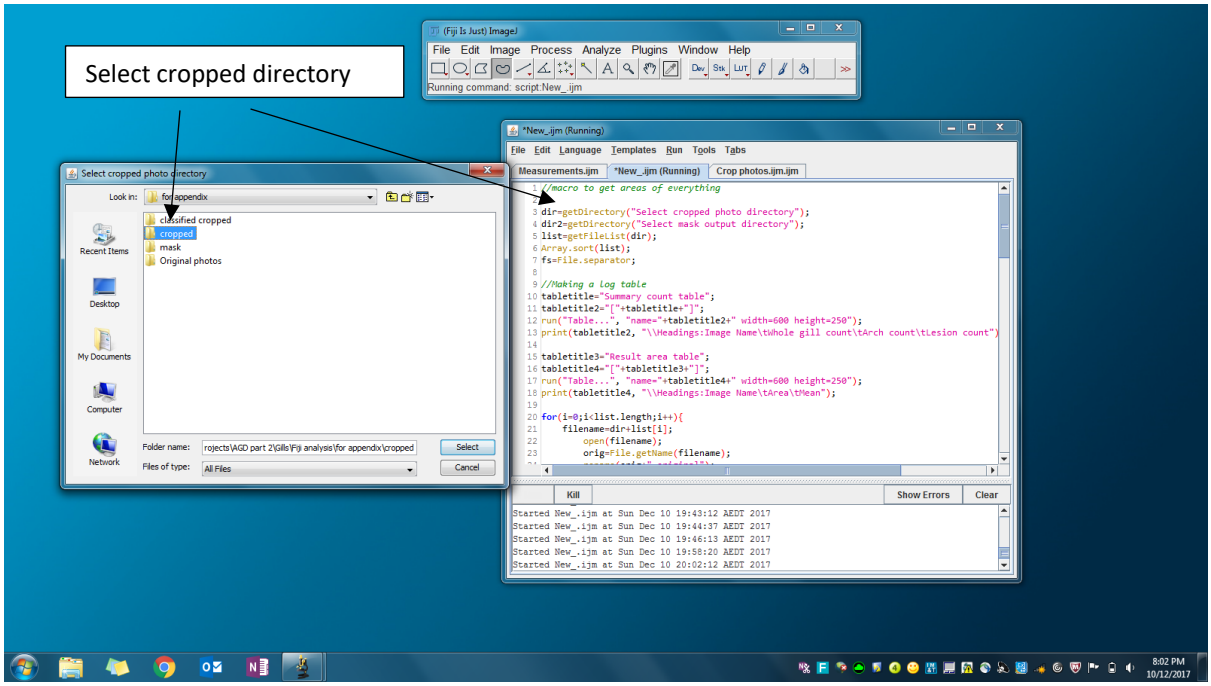

Figure A.7: User is prompted to select the cropped directory where the cropped photos were saved in the last macro.

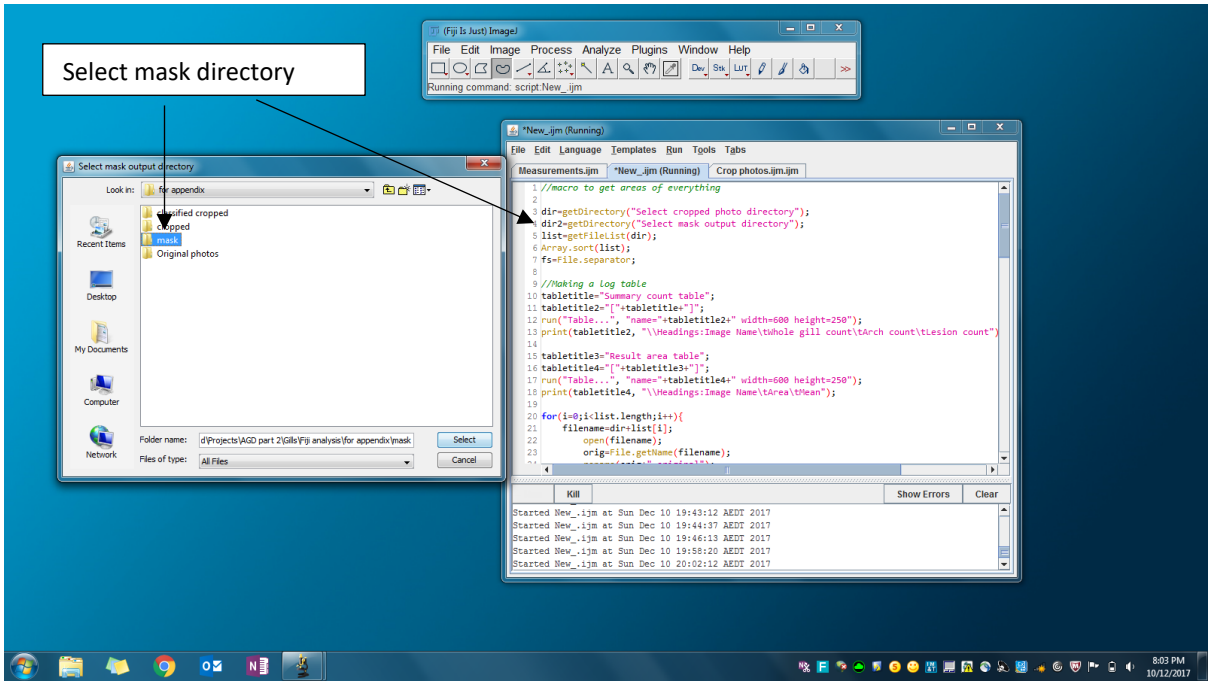

Figure A.8: The user is prompted to choose a folder to save the altered photos from this macro. In this case, the folder has been named 'mask'.

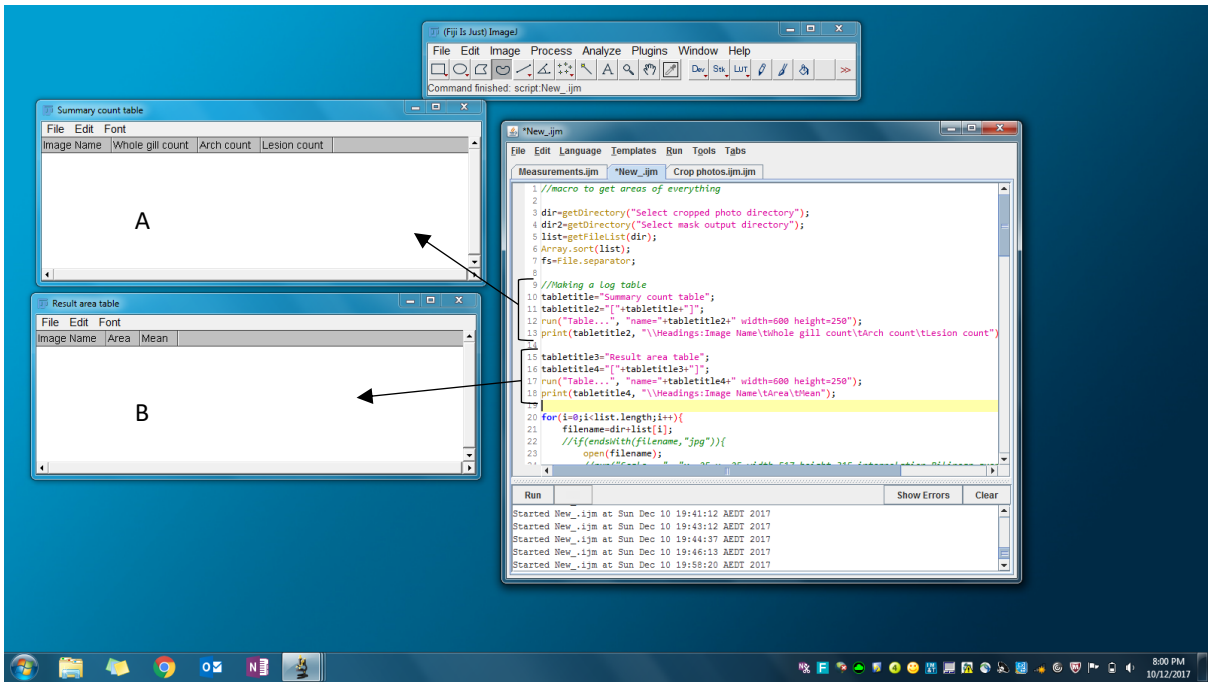

Figure A.9: Code that manually creates two data tables. (A) is a summary table with four columns: Image Name is the name of the image the data are measured from, Whole gill, Arch and Lesion count columns are the number of measurements taken for each. (B) is the results table where Image Name is the same as in (A), Area is the area of the measurement in pixels, and Mean is the mean pixel colour (from 0 = black to 255 = white).

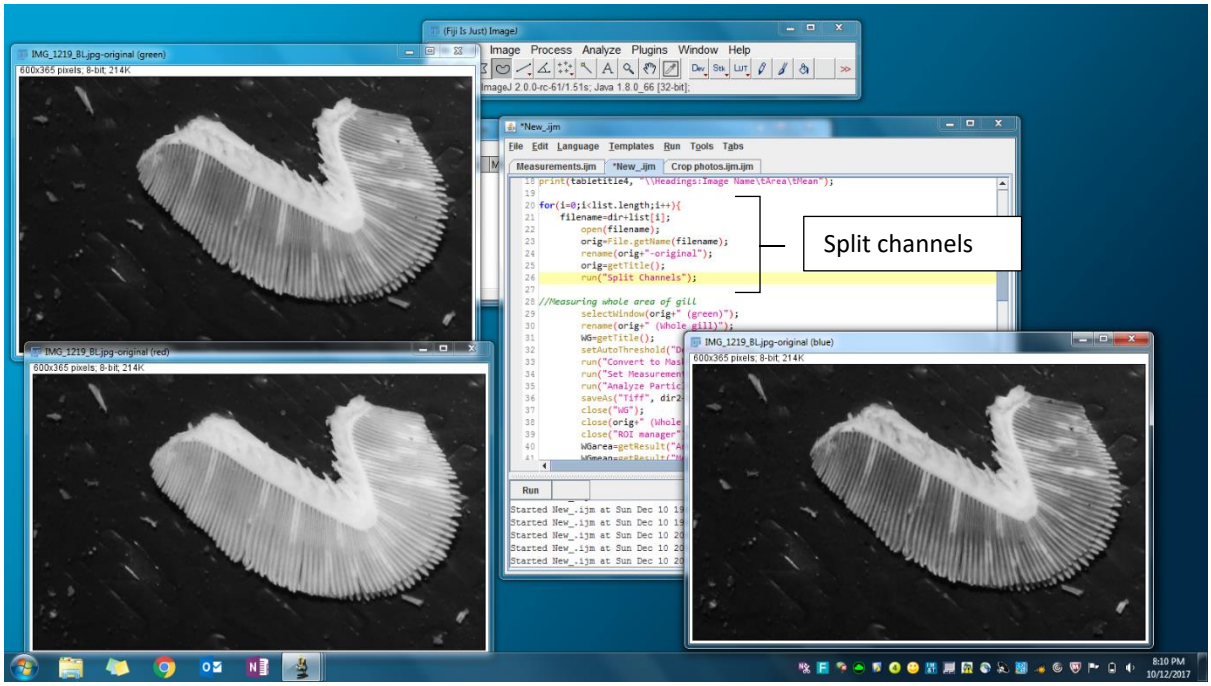

Figure A.10: The image is split into three 8-bit grayscale images containing the red, green, and blue components of the original.

Whole gill measurements

The first measurement is for the entire gill area which includes the filaments and the arch. The green channel is selected, the image renamed to include 'Whole gill', and a threshold is applied to the image using the default of a dark background (Fig. A.11). The image is then converted to a mask which is a binary image that the program can work with to automatically detect particles (Fig. A.12). Then the measurements of interest are specified: area (in pixels) and mean intensity (Fig. A.13). The area is the measurement of interest and the mean intensity allows the user to double check that the white area has been detected for measurement. Then the 'analyse particles' command is run. The user sets a size threshold of 20,000 – infinity pixels so that the program only detects particles larger than 20,000 pixels. The area measured is also outlined and the mask image saved in the mask folder specified earlier. Then the unneeded windows are closed. The results are then written to the 'Results area table' created manually earlier (Fig. A.14).

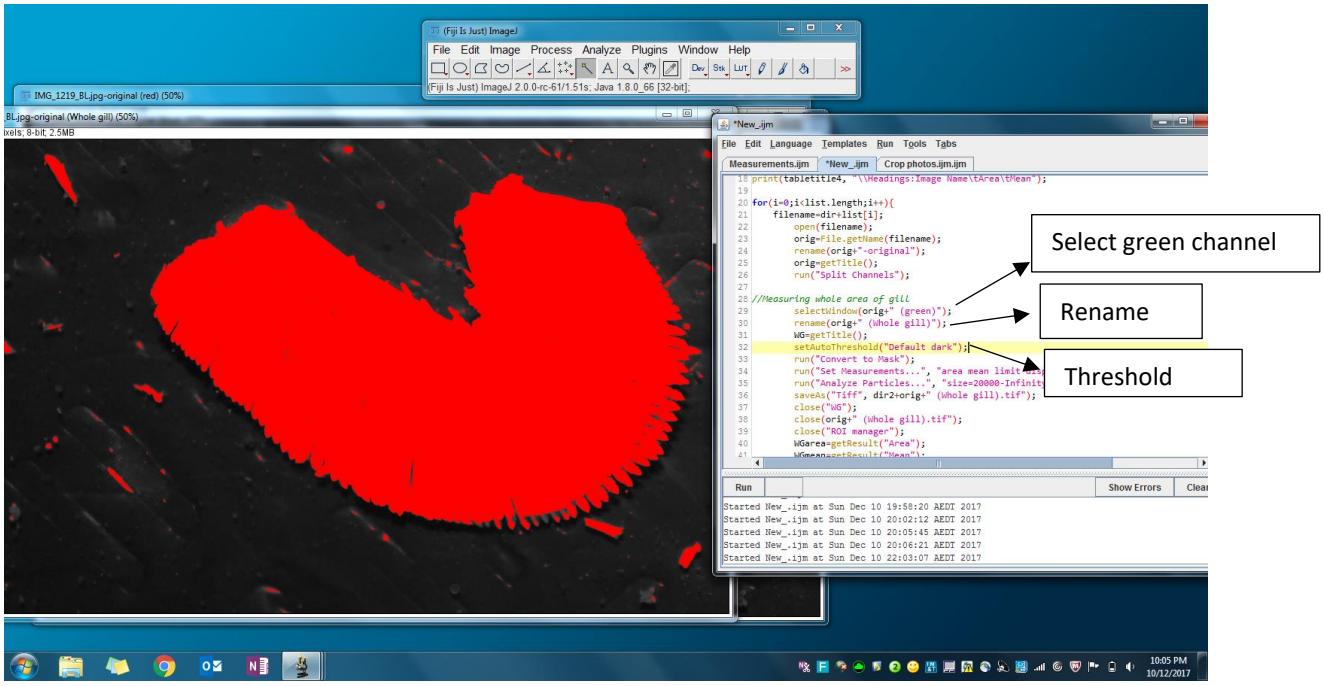

Figure A.11: The green channel is selected, renamed to append 'Whole gill' to the original name, and a colour threshold is run using the default of a dark background.

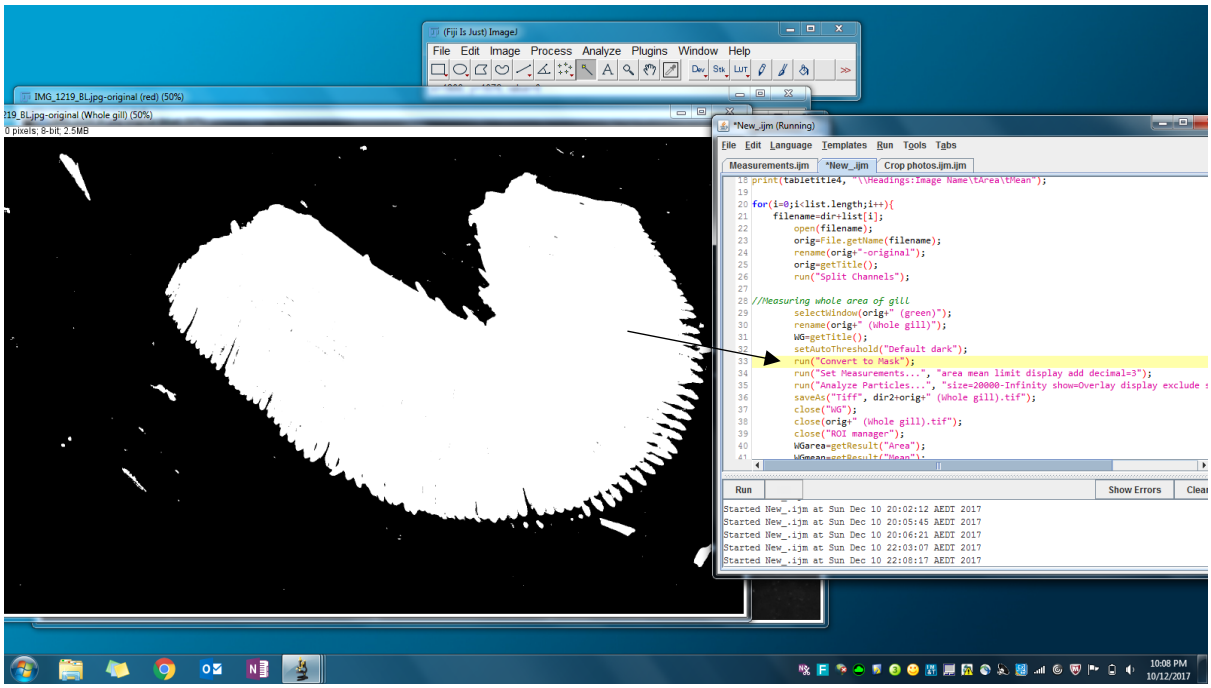

Figure A.12: The threshold image is converted to a mask which allows the program to recognise it as a 'particle'.

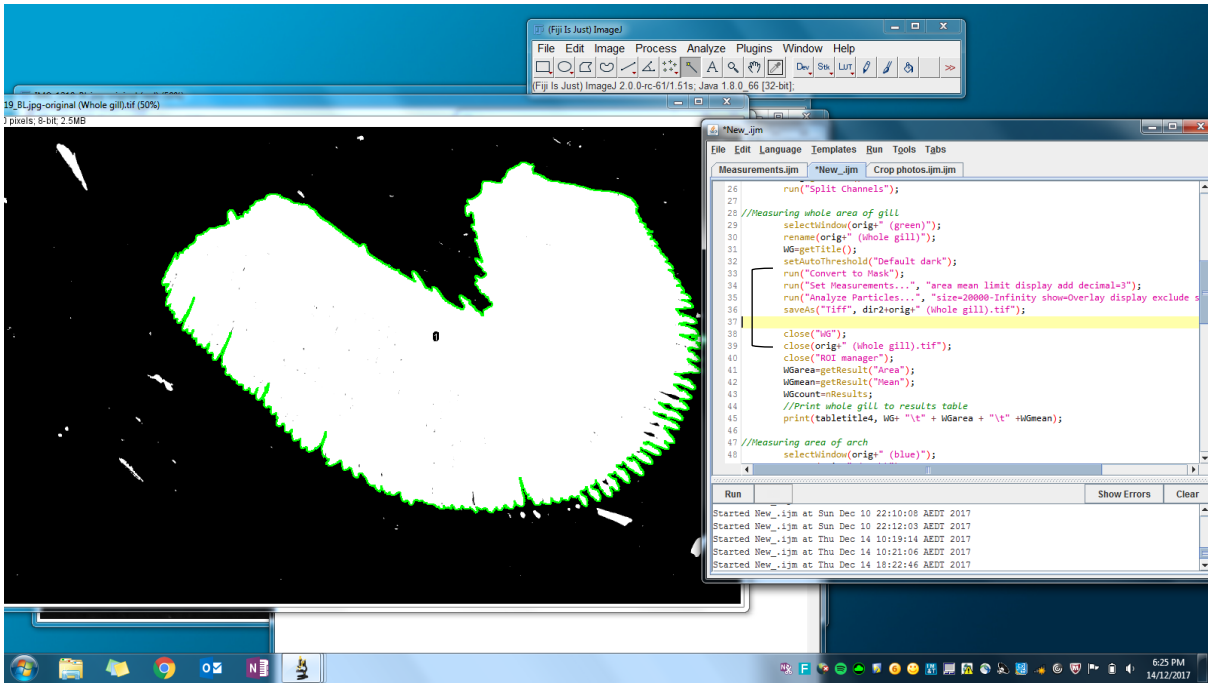

Figure A.13: This section of code sets the measurements to be taken (area and mean intensity of the region of interest), measures the region of interest (the highlighted area), saves the image into the mask folder, and closes unneeded windows.

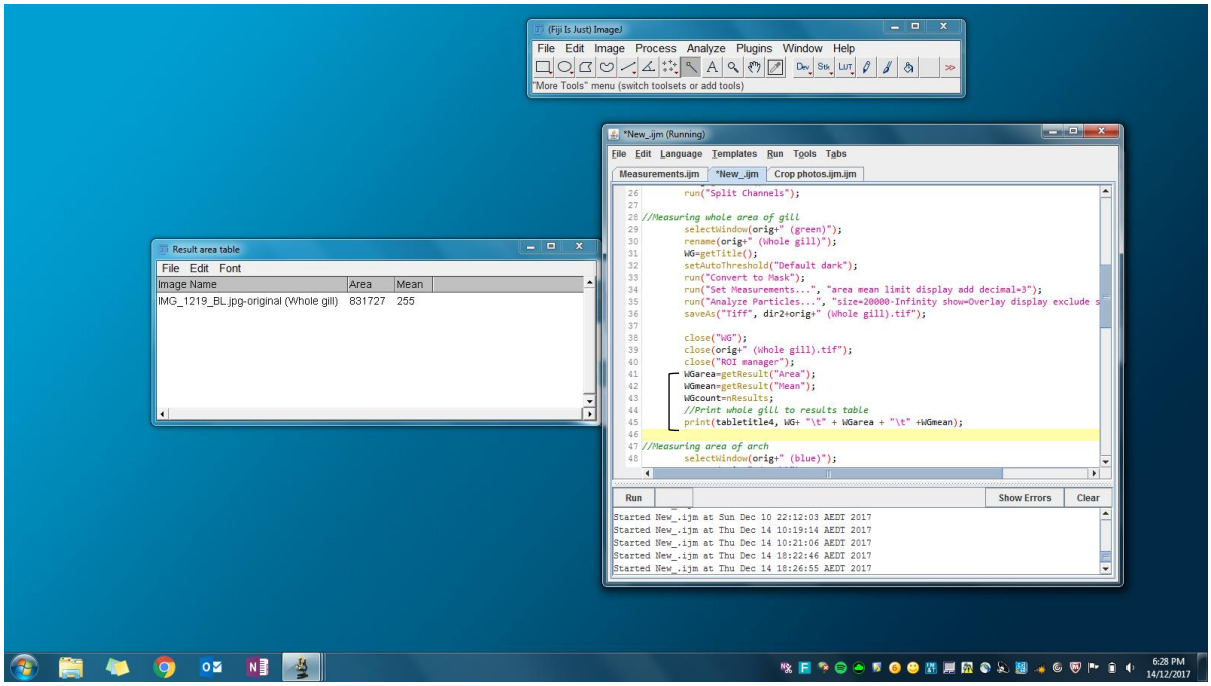

Figure A.14: This section of code writes the results to the manually created data table from Fig. A.**9**.

Arch measurements

The branchial arch then needs to be measured in order to subtract that area from the whole gill area to get the area of the filaments. The blue channel is selected and renamed to include 'Arch' (Fig. A.15). Then the user is prompted to highlight the arch using image threshold. This means adjusting the threshold levels to leave the arch free of colour. Once the user is satisfied with the thresholding, the user hits 'ok' and the macro moves on. The image is again converted to a mask (Fig. A.16), but this time the image mask needs to be inverted for the arch to appear white (Fig. A.17). Then the measurements can be conducted. Similar to the whole gill measurements, the measurements to be taken are specified (area and mean intensity), particles are analysed (again >20,000 pixels), the mask image is saved, and the results written to the 'Results area table' (Fig. A.18).

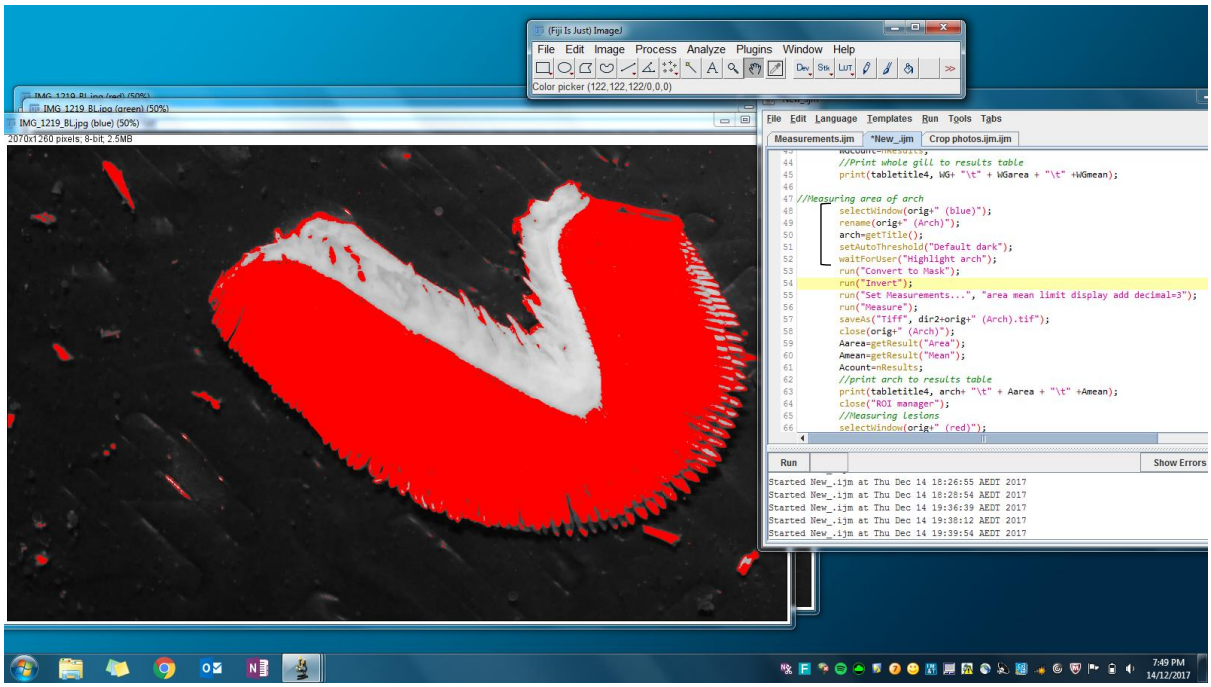

Figure A.15: The blue channel is selected and renamed to append 'Arch' to the name. A threshold is applied to the image and then the user is prompted to adjust the threshold so the arch is free of colour.

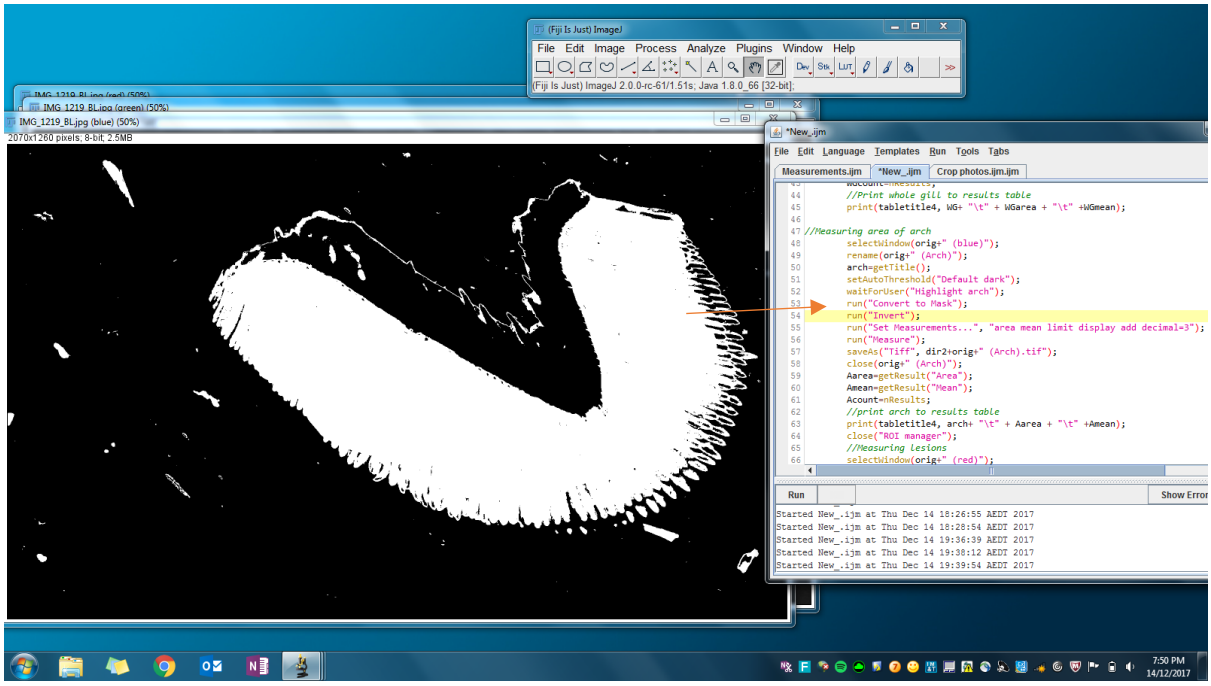

Figure A.16: The threshold image is converted to a mask.

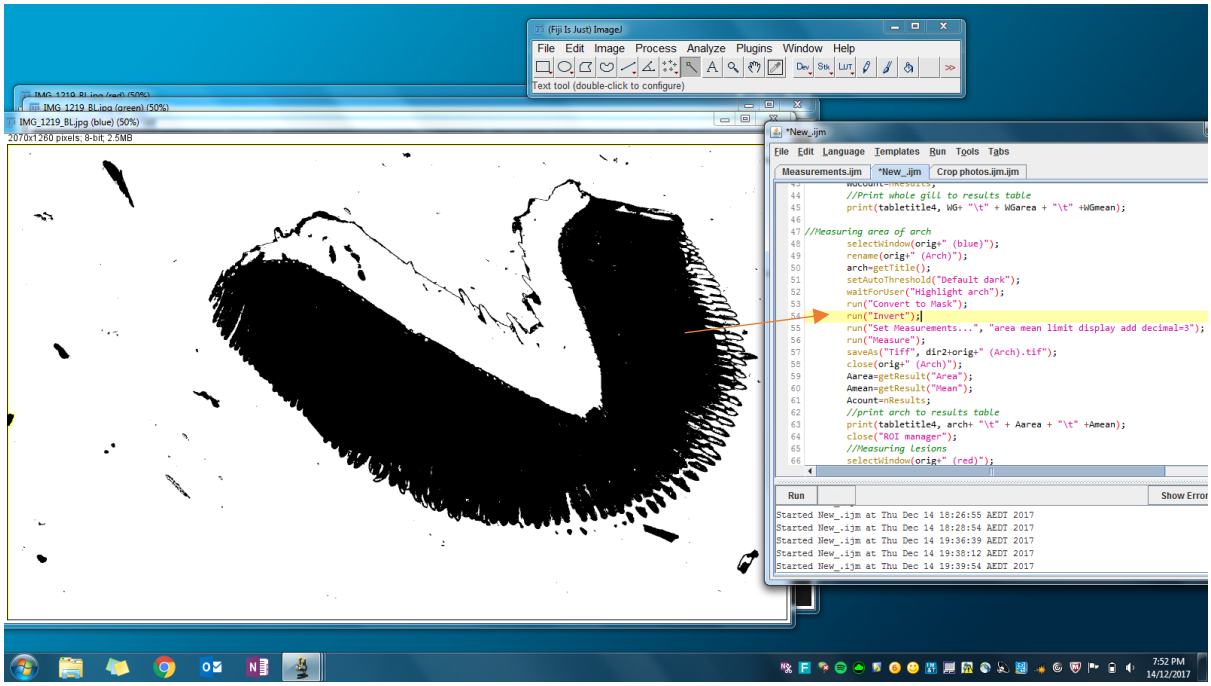

Figure A.17: This time the mask needs to be 'inverted' since the program picks up on the white sections of the image for the measurements.

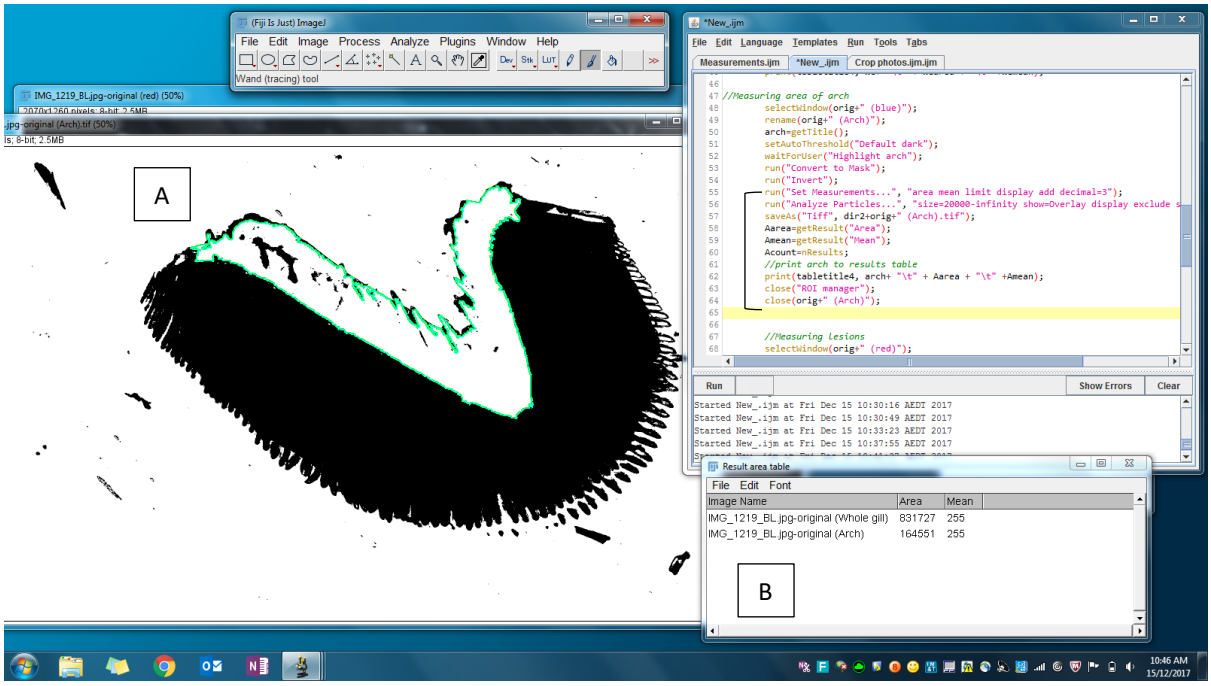

Figure A.18: The measurements are specified again (area and mean intensity), (A) the white area is measured in analyse particles, the image is saved into the mask folder, and (B) the result written to the data table.

Lesion measurements

The lesion measurements are conducted slightly differently. The red channel image is selected, renamed to include 'Lesions' and then the user is prompted to trace lesions and add them to the region of interest (ROI) manager (Fig. A.19). While tracing the lesions is more time consuming than using the 'analyse particles' method, the program does not always pick up the lesions accurately and more time is spent double checking what the program measured than just tracing them to begin with. After the user is satisfied with the traces, 'ok' is clicked so that the macro can move on. Again, the measurements are specified (area and mean intensity) and then the measurements are taken via the ROI manager. Finally, all measurements are added to the data tables (Fig. A.20). The 'summary table' that is created includes the n numbers of each section of measurements. The whole gill and arch measurements should only have 1 each and the user is able to double check that the measurements were taken correctly. The lesion count number is an easy way to see how many lesions each image contains. Lastly, another loop is created to add the lesion area data into the 'Results area table'. This loops through and adds each measurement one at a

time. Now that the user has reached the end of the macro, all open images are closed and the next image in the cropped directory is opened and the macro repeats itself.

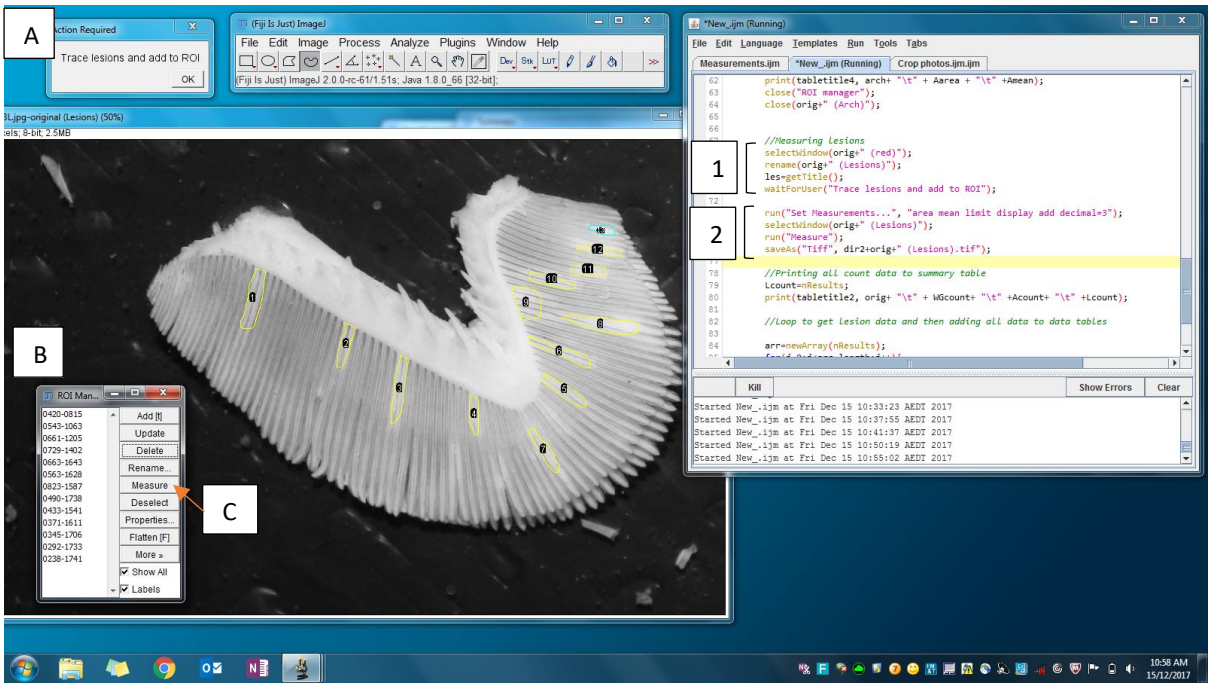

Figure A.19: (1) The red channel is selected and renamed to read 'Lesions'. (A) The user is prompted to trace the lesions and add the regions to the (B) region of interest (ROI) manager. (2) The measurements are set again (area and mean intensity) and then the ROIs are measured via the ROI manager (C).

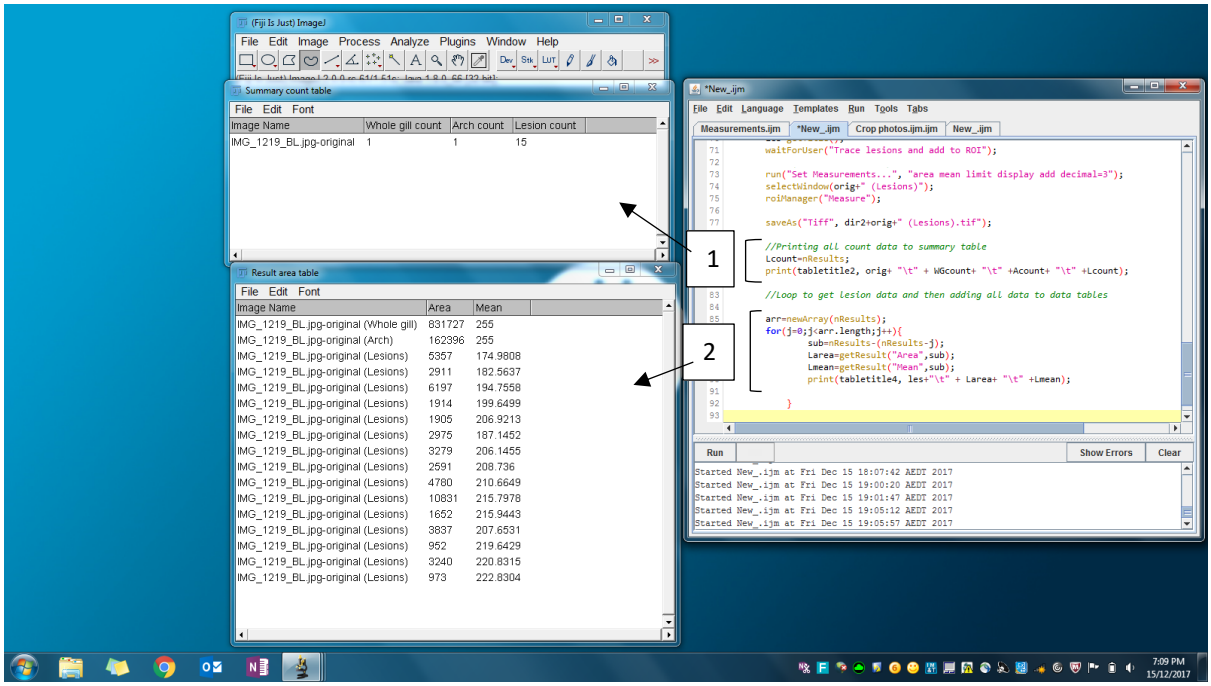

Figure A.20: (1) The n number of measurements of each section of the gill are added to the summary table. (2) A new loop of code is created to loop through the lesion measurements and add each one in turn to the result data table.

References

ABARES (2014). *Australian fisheries and aquaculture statistics 2013*. Research by the Australian Bureau of Agriculture and Resource Economics and Sciences.

Acharya, D. (2011). Fillet quality and yield of farmed Atlantic salmon (*Salmo salar* L.): variation between families, gender differences and the importance of maturation. Master's Thesis. Norwegian University of Life Sciences.

Adams, M. B. and Nowak, B. F. (2001). Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. *J. Fish Dis.* **24**, 535–542.

Adams, M. B. and Nowak, B. F. (2003). Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **26**, 601–614.

Adams, M. B. and Nowak, B. F. (2004). Sequential pathology after initial freshwater bath treatment for amoebic gill disease in cultured Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **27**, 163–173.

Adams, M. B., Crosbie, P. B. B. and Nowak, B. F. (2012). Preliminary success using hydrogen peroxide to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill disease (AGD). *J. Fish Dis.* **35**, 839–848.

Agnisola, C. and Tota, B. (1994). Structure and function of the fish cardiac ventricle: flexibility and limitations. *Cardioscience* **5**, 145–153.

Altimiras, J., Axelsoon, M., Claireaux, G., Lefrancois, C., Mercier, C. and Farrell, A. P. (2002). Cardiorespiratory status of triploid brown trout during swimming at two acclimation temperatures. *J. Fish Biol.* **60**, 102–116.

Andrewartha, S. J., Munns, S. L. and Edwards, A. (2016). Calibration of the HemoCue point-of-care analyser for determining haemoglobin concentration in a lizard and a fish. *Conserv. Physiol.* **4**, cow006.

Anttila, K., Dhillon, R. S., Boulding, E. G., Farrell, A. P., Glebe, B. D., Elliott, J. A. K., Wolters, W. R. and Schulte, P. M. (2013). Variation in temperature tolerance among families of Atlantic salmon (*Salmo salar*) is associated with hypoxia tolerance, ventricle size and myoglobin level. *J. Exp. Biol.* **216**, 1183–1190.

Arrhenius, S. (1896). XXXI.On the influence of carbonic acid in the air upon the temperature of the ground. *Philosophical Magazine Series 5* **41**, 237–276.

Atkins, M. E. and Benfey, T. J. (2008). Effect of acclimation temperature on routine metabolic rate in triploid salmonids. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **149**, 157–161.

Barnes, R. (Katersky), King, H. and Carter, C. G. (2011). Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar,* from a Tasmanian population. *Aquaculture* **318**, 397–401.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1**, 3–26.

Barton, B. A. (2011). Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. *N. AM. J. Aquacult*. **62**, 12-18.

Beamish, F. W. H. (1974). Apparent specific dynamic action of largemouth bass, *Micropterus salmoides*. *J. Rish. Res. Bd. Can.*

Beckenbach, A. T. (1975). Influence of body size and temperature on the critical oxygen tension of some Plethodontid salamanders. *Physiol. Zool.* **48**, 338–347.

Becker, C. D. and Genoway, R. G. (1979). Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environ. Biol. Fishes* **4**, 245–256.

Benfey, T. J. (1999). The physiology and behavior of triploid fishes. *Rev. Fish. Sci.* **7**, 39–67.

Benfey, T. (2001). Use of sterile triploid Atlantic salmon (*Salmo salar* L.) for aquaculture in New Brunswick, Canada. *ICES J. Mar. Sci.* **58**, 525–529.

Benfey, T. J. and Sutterlin, A. M. (1984a). The haematology of triploid landlocked Atlantic salmon, *Salmo solar* L. *J. Fish Biol.* **24**, 333–338.

Benfey, T. J. and Sutterlin, A. M. (1984b). Oxygen utilization by triploid landlocked Atlantic salmon (*Salmo salar* L.). *Aquaculture* **42**, 69–73.

Benfey, T. J., Sutterlin, A. M. and Thompson, R. J. (1984). Use of erythrocyte measurements to identify triploid salmonids. *Can. J. Fish. Aquat. Sci.* **41**, 980–984.

Benfey, T. J., McCabe, L. E., Pepin, P., Benfey, T., McCabe, L. and Pepin, P. (1997). Critical thermal maxima of diploid and triploid brook charr, *Salvelinus fontinalis*. *Environ. Biol. of Fish.* **49**, 259–264.

Bernier, N. J., Brauner, C. J., Heath, J. W. and Randall, D. J. (2004). Oxygen and carbon dioxide transport during sustained exercise in diploid and triploid chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* **61**, 1797–1805.

Bœuf, G. and Payan, P. (2001). How should salinity influence fish growth? *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **130**, 411–423.

Booth, J. H. (1978). The distribution of blood flow in the gills of fish: application of a new technique to rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **73**, 119–129.

Booth, J. H. (1979). Circulation in trout gills: the relationship between branchial perfusion and the width of the lamellar blood space. *Can. J. Zool.* **57**, 2183–2185.

Børsheim, E. and Bahr, R. (2003). Effect of exercise intensity, duration and mode on post-exercise oxygen consumption. *Sports Med.* **33**, 1037–1060.

Bradshaw, W. E. and Holzapfel, C. M. (2008). Genetic response to rapid climate change: it's seasonal timing that matters. *Mol. Ecol.* **17**, 157–166.

Brett, J. R. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Bd. Can.* **21**, 1183–1226.

Brett, J. R. (1971). Energetic responses of salmon to temperature- study of some thermal relations in physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *Am. Zool.* **11**, 99–113.

Brett, J. R. and Glass, N. R. (1973). Metabolic rates and critical swimming speeds of sockeye salmon (*Oncorhynchus nerka*) in relation to size and temperature. *J. Fish. Res. Bd. Can.* **30**, 379–387.

Bridle, A. R., Morrison, R. N. and Nowak, B. F. (2006a). The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during amoebic gill disease (AGD). *Fish Shellfish Immunol.* **20**, 346–364.

Bridle, A. R., Morrison, R. N., Cupit Cunningham, P. M. and Nowak, B. F. (2006b). Quantitation of immune response gene expression and cellular localisation of interleukin-1beta mRNA in Atlantic salmon, *Salmo salar* L., affected by amoebic gill disease (AGD). *Vet. Immunol. Immunopathol.* **114**, 121–134.

Brijs, J., Jutfelt, F., Clark, T. D., Gräns, A., Ekström, A. and Sandblom, E. (2015). Experimental manipulations of tissue oxygen supply do not affect warming tolerance of European perch. *J. Exp. Biol.* **218**, 2448–2454.

Bruneaux, M., Visse, M., Gross, R., Pukk, L., Saks, L. and Vasemägi, A. (2017). Parasite infection and decreased thermal tolerance: impact of proliferative kidney disease on a wild salmonid fish in the context of climate change. *Funct. Ecol.* **31**, 216–226.

Buckley, L. B., Hurlbert, A. H. and Jetz, W. (2012). Broad-scale ecological implications of ectothermy and endothermy in changing environments. *Global Ecol. Biogeogr.* **21**, 873–885.

Bustos, P. A., Young, N. D., Rozas, M. A., Bohle, H. M., Ildefonso, R. S., Morrison, R. N. and Nowak, B. F. (2011). Amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) farmed in Chile. *Aquaculture* **310**, 281–288.

Carline, R. F. and Machung, J. F. (2001). Critical thermal maxima of wild and domestic strains of trout. *Trans. Am. Fish. Soc.* **130**, 1211–1216.

Carter, C. G., McCarthy, I. D. and Houlihan, D. F. (1994). Food consumption, feeding behaviour, and growth of triploid and diploid Atlantic salmon, *Salmo salar* L., parr. *Can. J. Zool.* **72**, 609–617.

Castro, V., Grisdale-Helland, B., Jørgensen, S. M., Helgerud, J., Claireaux, G., Farrell, A. P., Krasnov, A., Helland, S. J. and Takle, H. (2013). Disease resistance is related to inherent swimming performance in Atlantic salmon. *BMC Physiol.* **13**, 1.

Claireaux, G. and Lefrançois, C. (2007). Linking environmental variability and fish performance: integration through the concept of scope for activity. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **362**, 2031–2041.

Claireaux, G., McKenzie, D. J., Genge, A. G., Chatelier, A., Aubin, J. and Farrell, A. P. (2005). Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J. Exp. Biol.* **208**, 1775–1784.

Clark, A. and Nowak, B. F. (1999). Field investigations of amoebic gill disease in Atlantic salmon, *Salmo salar* L., in Tasmania. *J. Fish Dis.* **22**, 433–443.

Clark, G., Powell, M. and Nowak, B. (2003). Effects of commercial freshwater bathing on reinfection of Atlantic salmon, *Salmo salar*, with amoebic gill disease. *Aquaculture* **219**, 135–142.

Clark, T. D., Sandblom, E., Cox, G. K., Hinch, S. G. and Farrell, A. P. (2008a). Circulatory limits to oxygen supply during an acute temperature increase in the Chinook salmon (*Oncorhynchus tshawytscha*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1631–9.

Clark, T. D., Eliason, E. J., Sandblom, E., Hinch, S. G. and Farrell, A. P. (2008b). Calibration of a hand-held haemoglobin analyser for use on fish blood. *J. Fish Biol.* **73**, 2587–2595.

Clark, T. D., Jeffries, K. M., Hinch, S. G. and Farrell, A. P. (2011). Exceptional aerobic scope and cardiovascular performance of pink salmon (*Oncorhynchus gorbuscha*) may underlie resilience in a warming climate. *J. Exp. Biol.* **214**, 3074–3081.

Clark, T. D., Donaldson, M. R., Pieperhoff, S., Drenner, S. M., Lotto, A., Cooke, S. J., Hinch, S. G., Patterson, D. A. and Farrell, A. P. (2012). Physiological benefits of being small in a changing world: responses of Coho salmon (*Oncorhynchus kisutch*) to an acute thermal challenge and a simulated capture event. *PLoS ONE* **7**, e39079.

Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771–2782.

Clark, T. D., Roche, D. G., Binning, S. A., Speers-Roesch, B. and Sundin, J. (2017). Maximum thermal limits of coral reef damselfishes are size-dependent and resilient to near-future ocean acidification. *J. Exp. Biol.* **220**, jeb–162529.

Clarke, A. and Johnston, N. M. (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *J. Anim. Ecol.* **68**, 893–905.

Cochrane, K., De Young, C., Soto, D., Bahri, T. and by, E. (2009). *Climate change implications for fisheries and aquaculture*. Rome: Food and Agriculture Organization of the United Nations.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J. L., Fichefet, T., Friedlingstein, P., GAo, X., Gutowski, W. J., Johns, T., Krinner, G., et al. (2013a). Chapter 12- Long-term climate change: projections, commitments and irreversibility. In *Climate Change 2013: The Physical Science Basis. IPCC Working Group 1 Contribution to AR5*, pp. 1029–1136. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Collins, G. M., Clark, T. D., Rummer, J. L. and Carton, A. G. (2013b). Hypoxia tolerance is conserved across genetically distinct sub-populations of an iconic, tropical Australian teleost (*Lates calcarifer*). *Conserv. Physiol.* **1**, cot029.

Cook, T., Folli, M., Klinck, J., Ford, S. and Miller, J. (1998). The relationship between increasing sea-surface temperature and the northward spread of *Perkinsus marinus* (Dermo) disease epizootics in oysters. *Estuar. Coast. Shelf. Sci.* **46**, 587–597.

Coucelo, J., Coucelo, J. and Azevedo, J. (1996). Ultrasonography characterization of heart morphology and blood flow of lower vertebrates. *J. Exp. Zool. A: Ecol. Gen. Physiol.* **275**, 73-82.

Currie, R. J., Bennett, W. A. and Beitinger, T. L. (1998). Critical thermal minima and maxima of three freshwater game-fish species acclimated to constant temperatures. *Environ. Biol. Fishes* 187–200.

Cutts, C. J., Betcalfe, N. B. and Caylor, A. C. (1998). Aggression and growth depression in juvenile Atlantic salmon: the consequences of individual variation in standard metabolic rate. *J. Fish Biol.* **52**, 1026–1037.

Daufresne, M., Lengfellner, K. and Sommer, U. (2009). Global warming benefits the small in aquatic ecosystems. *Proc. Natl. Acad. Sci. U S A* **106**, 12788–12793.

Davison, W., Franklin, C. E. and Carey, P. W. (1990). Oxygen uptake in the Antarctic teleost *Pagothenia borchgrevinki*. Limitations imposed by X-cell gill disease. *Fish. Physiol. Biochem.* **8**, 69–77.

Dejours, P. (1981). *Principles of comparative respiratory physiology*. sole distributors for the USA and Canada, Elsevier North-Holland.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. U S A* **105**, 6668–6672.

Douglas-Helders, M., Saksida, S., Raverty, S. and Nowak, B. F. (2001). Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon (*Salmo salar*). *B. Eur. Assoc. Fish Pat.* **21**, 114–116.

Duthie, G. G. and Hughes, G. M. (1987). The effects of reduced gill area and hyperoxia on the oxygen consumption and swimming speed of rainbow trout. *J. Exp. Biol.* **127**, 349–354.

Dyková, I., Figueras, A. and Novoa, B. (1995). Amoebic gill infection of turbot, *Scophthalmus maximus*. *Folia Parasitol.* **42**, 91–96.

Dyková, I., Figueras, A., Novoa, B. and Casal, J. F. (1998). Paramoeba sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Dis. Aquat. Org.* **33**, 137–141.

Dyková, I., Figueras, A. and Novoa, B. (1999). Epizoic amoebae from the gills of turbot *Scophthalmus maximus*. *Dis. Aquat. Org.* **38**, 33–38.

Dyková, I., Figueras, A. and Peric, Z. (2000). *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Dis. Aquat. Org.* **43**, 217–223.

Dyková, I., Nowak, B. F., Crosbie, P. B. B., Fiala, I., Pecková, H., Adams, M. B., Macháčková, B. and Dvořáková, H. (2005). *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *J. Fish Dis.* **28**, 49–64.

Ekström, A., Brijs, J., Clark, T. D., Gräns, A., Jutfelt, F. and Sandblom, E. (2016). Cardiac oxygen limitation during an acute thermal challenge in the European perch: effects of chronic environmental warming and experimental hyperoxia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **311**, R440–9.

Elliott, J. M. and Elliott, J. A. (1995). The effect of the rate of temperature increase on the critical thermal maximum for parr of Atlantic salmon and brown trout. *J. Fish Biol.* **47**, 917–919.

Elliott, J. M. and Elliott, J. A. (2010). Temperature requirements of Atlantic salmon, *Salmo salar*, brown trout, *Salmo trutta,* and Arctic charr, *Salvelinus alpinus*: predicting the effects of climate change. *J. Fish Biol.* **77**, 1793–1817.

Elliott, J. M. and Hurley, M. A. (1997). A functional model for maximum growth of Atlantic Salmon parr, *Salmo salar*, from two populations in northwest England. *Funct. Ecol.* **11**, 592–603.

Elliott, N. G. and Kube, P. D. (2009). Development and early results of the Tasmanian Atlantic salmon breeding program. *Aquaculture*.

Enss, M. L., Cornberg, M., Wagner, S., Gebert, A., Henrichs, M., Eisenblätter, R., Beil, W., Kownatzki, R. and Hedrich, H. J. (2000). Proinflammatory cytokines trigger MUC gene expression and mucin release in the intestinal cancer cell line LS180. *Inflamm. Res.* **49**, 162–169.

Ern, R., Norin, T., Gamperl, A. K. and Esbaugh, A. J. (2016). Oxygen dependence of upper thermal limits in fishes. *J. Exp. Biol.* **219**, 3376–3383.

Evans, D. O. (1990). Metabolic thermal compensation by rainbow trout: effects on standard metabolic rate and potential usable power. *Trans. Am. Fish. Soc.* **119**, 585–600.

FAOSTAT (2015). *Food and Agriculture Organization of the United Nations*.

Farrell, A. P. (2009). Environment, antecedents and climate change: lessons from the study of temperature physiology and river migration of salmonids. *J. Exp. Biol.* **212**, 3771–3780.

Farrell, A. P., Sobin, S. S., Randall, D. J. and Crosby, S. (1980). Intralamellar blood flow patterns in fish gills. *Am. J. Physiol.* **239**, R428–36.

Farrell, A. P., Hammons, A. M., Graham, M. S. and Tibbits, G. F. (1988). Cardiac growth in rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **66**, 2368–2373.

Fernandes, M. N. and Rantin, F. T. (1989). Respiratory responses of *Oreochromis niloticus* (Pisces, Cichlidae) to environmental hypoxia under different thermal conditions. *J. Fish Biol.* **35**, 509–519.

Fiala, I. and Dyková, I. (2003). Molecular characterisation of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus*. *Dis. Aquat. Org.* **55**, 11–16.

Findlay, V. L. and Munday, B. L. (1998). Further studies on acquired resistance to amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **21**, 121–125.

Findlay, V. L., Helders, M., Munday, B. L. and Gurney, R. (1995). Demonstration of resistance to reinfection with Paramoeba sp. by Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **18**, 639–642.

Findlay, V. L., Zilberg, D. and Munday, B. L. (2000). Evaluation of levamisole as a treatment for amoebic gill disease of Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **23**, 193–198.

Finstad, A. G., Naesje, T. F. and Forseth, T. (2004). Seasonal variation in the thermal performance of juvenile Atlantic salmon (*Salmo salar*). *Freshwater Biol.* **49**, 1459–1467.

Fisk, D. M., Powell, M. D. and Nowak, B. F. (2002). The effect of amoebic gill disease and hypoxia on survival and metabolic rate of Atlantic salmon (*Salmo salar*). *B. Eur. Assoc. Fish Pat.* **22**, 190–194.

Ford, S. E. (1996). Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? *J. Shellfish Res.* **15**, 45–56.

Forseth, T., Hurley, M. A., Jensen, A. J. and Elliott, J. M. (2001). Functional models for growth and food consumption of Atlantic salmon parr, *Salmo salar*, from a Norwegian river. *Freshwater Biol.* **46**, 173–186.

Fox, J. and Weisberg, S. (2011). *An R Companion to Applied Regression*. 2nd ed. Thousand Oaks, California: Sage.

Franklin, C. E. and Axelsson, M. (1994). Coronary hemodynamics in elasmobranchs and teleosts. *Cardioscience* **5**, 155–161.

Franklin, C. E. and Davie, P. S. (1992). Dimensional analysis of the ventricle of an *in situ* perfused trout heart using echocardiography. *J. Exp. Biol.* **166**, 47–60.

Fraser, T. W. K., Fjelldal, P. G., Hansen, T. and Mayer, I. (2012). Welfare considerations of triploid fish. *Rev. Fish. Sci.* **20**, 192–211.

Fraser, T. W. K., Hansen, T., Skjæraasen, J. E., Mayer, I., Sambraus, F. and Fjelldal, P. G. (2013). The effect of triploidy on the culture performance, deformity prevalence, and heart morphology in Atlantic salmon. *Aquaculture* **416-417**, 255–264.

Fraser, T. W. K., Fleming, M. S., Poppe, T. T., Hansen, T. and Fjelldal, P. G. (2014). The effect of ploidy and incubation temperature on survival and the prevalence of aplasia of the septum transversum in Atlantic salmon, Salmo salar L. *J. Fish Dis.* **37**, 189–200.

Fraser, T. W. K., Mayer, I., Hansen, T., Poppe, T. T., Skjaeraasen, J. E., Koppang, E. O. and Fjelldal, P. G. (2015). Vaccination and triploidy increase relative heart weight in farmed Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **38**, 151–160.

Frederich, M. and Pörtner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279**, R1531–8.

Frusher, S. D., Hobday, A. J., Jennings, S. M., Creighton, C., D'Silva, D., Haward, M., Holbrook, N. J., Nursey-Bray, M., Pecl, G. T. and van Putten, E. I. (2013). The short history of research in a marine climate change hotspot: from anecdote to adaptation in south-east Australia. *Rev. Fish Biol. Fish.* **24**, 593-611.

Fry, F. E. (1947). *Effects of the environment on animal activity*. Toronto: University of Toronto Press.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. In *Environmental Relations and Behavior*, pp. 1–98. Elsevier.

Fu, S. J., Zeng, L. Q., Li, X. M., Pang, X., Cao, Z. D., Peng, J. L. and Wang, Y. X. (2009). Effect of meal size on excess post-exercise oxygen consumption in fishes with different locomotive and digestive performance. *J. Comp. Physiol. B.: Biochem. Syst. Environ. Physiol.* **179**, 509–517.

Fulton, T. W. (1904). The rate of growth of fishes. *Fisheries Board of Scotland, Annual Report 22(Part 3)* 141–241.

Furspan, P., Prange, H. D., and Greenwald, L. (1984). Energetics and osmoregulation in the catfish, Ictalurus nebulosus and I. punctatus. *Comp Biochem. Physiol. Part A.* **77**, 773-778.

Gaesser, G. A. and Brooks, G. A. (1984). Metabolic bases of excess post-exercise oxygen consumption: a review. *Med. Sci. Sports Exerc.* **16**, 29–43.

Galbreath, P. F., Jean, W. S., Anderson, V. and Thorgaard, G. H. (1994). Freshwater performance of all-female diploid and triploid Atlantic salmon. *Aquaculture* **128**, 41–49.

Galbreath, P. F., Adams, N. D., Sherrill, L. W. and Martin, T. H. (2006). Thermal tolerance of diploid versus triploid rainbow trout and brook trout assessed by time to chronic lethal maximum. *Environ. Biol. Fishes* **75**, 183–193.

Gallaugher, P. and Farrell, A. P. (1998). Hematocrit and Blood Oxygen-Carrying Capacity. In *Fish Respiration*, pp. 185–227. Elsevier.

Galloway, B. J. and Kieffer, J. D. (2003). The effects of an acute temperature change on the metabolic recovery from exhaustive exercise in juvenile Atlantic salmon (*Salmo salar*). *Physiol. Biochem. Zool.* **76**, 652–662.

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M. and Charnov, E. L. (2001). Effects of size and temperature on metabolic rate. *Science* **293**, 2248–2251.

Gleeson, T. T. and Hancock, T. V. (2002). Metabolic implications of a “run now, pay later” strategy in lizards: an analysis of post-exercise oxygen consumption. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **133**, 259–267.

Gollock, M. J., Currie, S., Petersen, L. H. and Gamperl, A. K. (2006). Cardiovascular and haematological responses of Atlantic cod (*Gadus morhua*) to acute temperature increase. *J. Exp. Biol.* **209**, 2961–2970.

Graham, M. S. and Farrell, A. P. (1989). The effect of temperature acclimation and adrenaline on the performance of a perfused trout heart. *Physiol. Zool.* **62**, 38–61.

Graham, M. S. and Farrell, A. P. (1992). Environmental influences on cardiovascular variables in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.* **41**, 851–858.

Graham, M. S., Fletcher, G. L. and Benfey, T. J. (1985). Effect of triploidy on blood oxygen content of Atlantic salmon. *Aquaculture* **50**, 133–139.

Grande, M. and Andersen, S. (1991). Critical thermal maxima for young salmonids. *J. Freshw. Ecol.* **6**, 275–279.

Guderley, H. and Pörtner, H. O. (2010). Metabolic power budgeting and adaptive strategies in zoology: examples from scallops and fish. *Can. J. Zool.* **88**, 753–763.

Hanna, S. K., Haukenes, A. H., Foy, R. J. and Buck, C. L. (2008). Temperature effects on metabolic rate, swimming performance and condition of Pacific cod *Gadus macrocephalus* Tilesius. *J. Fish Biol.* **72**, 1068–1078.

Harris, J. O., Powell, M. D., Attard, M. G. and DeHayr, L. (2005). Clinical assessment of chloramine-T and freshwater as treatments for the control of gill amoebae in Atlantic salmon, *Salmo salar* L. *Aquac. Res.* **36**, 776–784.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S. and Samuel, M. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158–2162.

Helmuth, B., Kingsolver, J. G. and Carrington, E. (2005). Biophysics, physiological ecology, and climate change: does mechanism matter? *Annu. Rev. Physiol.* **67**, 177–201.

Henry, J. A. C. and Houston, A. H. (1984). Absence of respiratory acclimation to diurnally-cycling temperature conditions in rainbow trout. *Comp. Biochem. Physiol. A: Physiol.* **77**, 727–734.

Hoar, W. S. (1988). 4 The Physiology of Smolting Salmonids. In *Fish physiology*, Academic Press, **11**, 275–343.

Hobday, A. J. and Pecl, G. T. (2014). Identification of global marine hotspots: sentinels for change and vanguards for adaptation action. *Rev. Fish Biol. Fish.* **24**, 415–425.

Houston, A. I., McNamara, J. M., Barta, Z. and Klasing, K. C. (2007). The effect of energy reserves and food availability on optimal immune defence. *Proc. Biol. Sci.* **274**, 2835–2842.

Howard, T. and Carson, J. (1993). Verification that *Paramoeba* species are consistently associated with gill damage in fish affected with amoebic gill disease. *Proceedings of the Saltas Research and Development Review Seminar*.

Hvas, M., Karlsbakk, E., Mæhle, S., Wright, D. W. and Oppedal, F. (2017a). The gill parasite *Paramoeba perurans* compromises aerobic scope, swimming capacity and ion balance in Atlantic salmon. *Conserv. Physiol.* **5**, cox066.

Hvas, M., Folkedal, O., Imsland, A. and Oppedal, F. (2017b).The effect of thermal acclimation on aerobic scope and critical swimming speed in Atlantic salmon, *Salmo salar. J. Exp. Biol.* **220**, 2757-2764.

Hwang, P.-P., Lee, T.-H. and Lin, L.-Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R28–47.

Hyndman, C. A., Kieffer, J. D. and Benfey, T. J. (2003). Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture* **221**, 629–643.

Iwama, G. K. (1996a). Growth of salmonids. In *Principles of Salmonid Culture*, pp. 467–515. Elsevier.

Iwama, G. K. (1996b). Chapter 7 - Growth of Salmonids. In *Developments in Aquaculture and Fisheries Science* **29**, 467–515.

Johansen, I. B., Sandblom, E., Skov, P. V., Gräns, A., Ekström, A., Lunde, I. G., Vindas, M. A., Zhang, L., Höglund, E., Frisk, M., et al. (2017). Bigger is not better: cortisol-induced cardiac growth and dysfunction in salmonids. *J. Exp. Biol.* **220**, 2545–2553.

Johnson, O. W., Dickhoff, W. W. and Utter, F. M. (1986). Comparative growth and development of diploid and triploid coho salmon, *Oncorhynchus kisutch*. *Aquaculture* **57**, 329–336.

Jones, D. R. (1982). Anaerobic exercise in teleost fish. *Can. J. Zool.* **60**, 1131–1134.

Jones, M. A., Powell, M. D., Becker, J. A. and Carter, C. G. (2007). Effect of an acute necrotic bacterial gill infection and feed deprivation on the metabolic rate of Atlantic salmon *Salmo salar*. *Dis. Aquat. Org.* **78**, 29–36.

Jonsson, B., Forseth, T., Jensen, A. J. and Naesje, T. F. (2001). Thermal performance of juvenile Atlantic Salmon, *Salmo salar* L. *Funct. Ecol.* **15**, 701–711.

Jungalwalla, P. J. (1991). Production of non-maturing Atlantic salmon in Tasmania. *Canadian technical report of fisheries and aquatic sciences*. 1789, 47-71.

Karvonen, A., Rintamäki, P., Jokela, J. and Valtonen, E. T. (2010). Increasing water temperature and disease risks in aquatic systems: climate change increases the risk of some, but not all, diseases. *Int. J. Parasitol.* **40**, 1483–1488.

Kearney, M. and Porter, W. P. (2004). Mapping the fundamental niche: physiology, climate, and the distribution of a nocturnal lizard. *Ecology* **85**, 3119–3131.

Kent, M. L., Sawyer, T. K. and Hedrick, R. P. (1988). *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in sea water. *Dis. Aquat. Org.* **5**, 163–169.

Kiceniuk, J. W. and Jones, D. R. (1977). The oxygen transport system in trout (Salmo gairdneri) during sustained exercise. *J. Exp. Biol.* **69**, 247–260.

Kieffer, J. D. and Tufts, B. L. (1996). The influence of environmental temperature on the role of the rainbow trout gill in correcting the acid-base disturbance following exhaustive exercise. *Physiol. Zool.* **69**, 1301–1323.

Killen, S. S., Norin, T. and Halsey, L. G. (2017). Do method and species lifestyle affect measures of maximum metabolic rate in fishes? *J. Fish Biol.* **90**, 1037–1046.

Kirtman, B., Power, S., Adedoyin, J. A., Boer, G. J., Bojariu, R., Camilloni, I., Doblas-Reyes, F. J., Fiore, A. M., Kimoto, M., Meehl, F. A., et al. (2013). Chapter 11- Near-term climate change: projections and predictability. In *Climate Change 2013: The Physical Science Basis. IPCC Working Group 1 Contribution to AR5.*, pp. 953–1028. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Klaiman, J. M., Fenna, A. J., Shiels, H. A., Macri, J. and Gillis, T. E. (2011). Cardiac remodeling in fish: strategies to maintain heart function during temperature Change. *PLoS ONE* **6**, e24464.

Kleiber, M. (1961). The fire of life. An introduction to animal energetics. *The fire of life. An introduction to animal energetics.*

Kube, P. D., Taylor, R. S. and Elliott, N. G. (2012). Genetic variation in parasite resistance of Atlantic salmon to amoebic gill disease over multiple infections. *Aquaculture* **364-365**, 165–172.

Langdon, J. S. (1990). Major protozoan and metazoan parasitic diseases of Australian finfish. *Fin Fish Diseases: Refresher Course for Veterinarians* 233–255.

Lannig, G., Bock, C., Sartoris, F. J. and Pörtner, H. O. (2004). Oxygen limitation of thermal tolerance in cod, *Gadus morhua* L., studied by magnetic resonance imaging and on-line venous oxygen monitoring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287**, R902–10.

Leclercq, E., Taylor, J. F., Fison, D., Fjelldal, P. G., Diez-Padrisa, M., Hansen, T. and Migaud, H. (2011). Comparative seawater performance and deformity prevalence in out-of-season diploid and triploid Atlantic salmon (Salmo salar) post-smolts. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **158**, 116–125.

Lee, C. G., Farrell, A. P., Lotto, A., MacNutt, M. J., Hinch, S. G. and Healey, M. C. (2003a). The effect of temperature on swimming performance and oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon stocks. *J. Exp. Biol.* **206**, 3239–3251.

Lee, C. G., Farrell, A. P., Lotto, A., Hinch, S. G. and Healey, M. C. (2003b). Excess post-exercise oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon following critical speed swimming. *J. Exp. Biol.* **206**, 3253–3260.

Leef, M. J., Harris, J. O. and Powell, M. D. (2005a). Respiratory pathogenesis of amoebic gill disease (AGD) in experimentally infected Atlantic salmon *Salmo salar*. *Dis. Aquat. Org.* **66**, 205–213.

Leef, M. J., Harris, J. O., Hill, J. and Powell, M. D. (2005b). Cardiovascular responses of three salmonid species affected with amoebic gill disease (AGD). *J. Comp. Physiol. B: Biochem. Syst. Environ. Physiol.* **175**, 523–532.

Leef, M. J., Harris, J. O. and Powell, M. D. (2007a). The respiratory effects of chloramine-T exposure in seawater acclimated and amoebic gill disease-affected Atlantic salmon *Salmo salar* L. *Aquaculture* **266**, 77–86.

Leef, M. J., Hill, J. V., Harris, J. O. and Powell, M. D. (2007b). Increased systemic vascular resistance in Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. *J. Fish Dis.* **30**, 601–613.

Leef, M. J., Harris, J. O. and Powell, M. D. (2007c). Metabolic effects of amoebic gill disease (AGD) and chloramine-T exposure in seawater-acclimated Atlantic salmon *Salmo salar*. *Dis. Aquat. Org.* **78**, 37–44.

Lefevre, S. (2016). Are global warming and ocean acidification conspiring against marine ectotherms? A meta-analysis of the respiratory effects of elevated temperature, high CO2 and their interaction. *Conserv. Physiol.* **4**, cow009.

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *J Stat Softw* **69**, 1–33.

Lijalad, M. and Powell, M. D. (2009). Effects of lower jaw deformity on swimming performance and recovery from exhaustive exercise in triploid and diploid Atlantic salmon *Salmo salar* L. *Aquaculture* **290**, 145–154.

Lund, S. G., Caissie, D., Cunjak, R. A., Vijayan, M. M. and Tufts, B. L. (2002). The effects of environmental heat stress on heat-shock mRNA and protein expression in Miramichi Atlantic salmon (*Salmo salar*) parr. *Can. J. Fish. Aquat. Sci.* **59**, 1553–1562.

Lutterschmidt, W. I. and Hutchison, V. H. (1997). The critical thermal maximum: history and critique. *Can. J. Zool.* **75**, 1561–1574.

Magozzi, S. and Calosi, P. (2015). Integrating metabolic performance, thermal tolerance, and plasticity enables for more accurate predictions on species vulnerability to acute and chronic effects of global warming. *Glob. Chang. Biol.* **21**, 181–194.

Maxime, V. (2008). The physiology of triploid fish: current knowledge and comparisons with diploid fish. *Fish Fish.* **9**, 67–78.

McCarthy, I. D., Carter, C. G., Houlihan, D. F., Johnstone, R. and Mitchell, A. I. (1996). The performance of all-female diploid and triploid Atlantic salmon smolts on transfer together to sea water. *J. Fish Biol.* **48**, 545–548.

McCormick, S. D., Moyes, C. D. and Ballantyne, J. S. (1989). Influence of salinity on the energetics of gill and kidney of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **6**, 243–254.

McDonald, D. G. (1983). The effects of H^+ upon the gills of freshwater fish. *Can. J. Zool.* **61**, 691–703.

McGeachy, S. A., Benfey, T. J. and Friars, G. W. (1995). Freshwater performance of triploid Atlantic salmon (*Salmo salar*) in New Brunswick aquaculture. *Aquaculture* **137**, 333–341.

Messmer, V., Pratchett, M. S., Hoey, A. S., Tobin, A. J., Coker, D. J., Cooke, S. J. and Clark, T. D. (2016). Global warming may disproportionately affect larger adults in a predatory coral reef fish. *Glob. Chang. Biol.* **23**, 2230–2240.

Metcalfe, N. B., Taylor, A. C. and Thorpe, J. E. (1995). Metabolic rate, social status and life-history strategies in Atlantic salmon. *An. Behav.* **49**, 431–436.

Meyer, F. P. (1991). Aquaculture disease and health management. *J. Anim. Sci.* **69**, 4201–4208.

Milligan, C. L. (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol. A: Physiol.* **113**, 51–60.

Milligan, C. L. and Wood, C. M. (1987). Regulation of blood oxygen transport and red cell pHi after exhuastive activity in rainbow trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus*). *J. Exp. Biol.* **133**, 263–282.

Morgan, J. D. and Iwama, G. K. (1991). Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* **48**, 2083–2094.

Morgan, J. D. and Iwama, G. K. (1999). Energy cost of NaCl transport in isolated gills of cutthroat trout. Am. J. Physiol-Reg. I. **277**, R631-R639.

Morgan, J. D. and Iwama, G. K. (1998). Salinity effects on oxygen consumption, gill Na+, K+-ATPase and ion regulation in juvenile coho salmon. *J. Fish Biol.* **53**, 1110–1119.

Morrison, R. N., Crosbie, P. B. B. and Nowak, B. F. (2004). The induction of laboratory-based amoebic gill disease revisited. *J. Fish Dis.* **27**, 445–449.

Morrison, R. N., Cooper, G. A., Koop, B. F., Rise, M. L., Bridle, A. R., Adams, M. B. and Nowak, B. F. (2006). Transcriptome profiling the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.): a role for tumor suppressor p53 in AGD pathogenesis? *Physiol. Genomics* **26**, 15–34.

Muggeo, V. M. R. (2003). Estimating regression models with unknown break-points. *Stat. Med.* **22**, 3055–3071.

Munday, B. L. (1986). Diseases of salmonids. In *Proceedings of the workshop on diseases of Australian fish and shellfish*, 127–141.

Munday, B. L., Foster, C. K., Roubal, F. R. and Lester, R. J. G. (1990). Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar* and rainbow trout, *Salmo gairdneri* in Tasmania. In *Proceedings of the Third International Colloquium on Pathology in Marine Aquaculture, held in Gloucester Point, Virginia, USA, October 2-6. 1988* (ed. Perkins, F. O. and Cheng, T. C.), pp. 215–222. Academic Press Inc.

Munday, B. L., Zilberg, D. and Findlay, V. (2001). Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. *J. Fish Dis.* **24**, 497–507.

Nordlie, F. G. (1978). The influence of environmental salinity on respiratory oxygen demands in the euryhaline teleost, Ambassis interrupta Bleeker. *Comp. Biochem. Physiol. Part A*. **59**, 271-274.

Nordlie, F. G. and Leffler, C. W. (1975). **I**onic regulation and the energetics of osmoregulation in Mugil cephalus Lin. *Comp. Biochem. Physiol. Part A*. **51,** 125-131.

Nordlie, F. G., Walsh, S. J., Haney, D. C., and Nordlie, T. F. (1991). The influence of ambient salinity on routine metabolism in the teleost Cyprinodon variegatus Lacepede. *J Fish Biol*. **38**, 115-122.

Norin, T. and Clark, T. D. (2016). Measurement and relevance of maximum metabolic rate in fishes. *J. Fish Biol.* **88**, 122–151.

Norin, T., Malte, H. and Clark, T. D. (2014). Aerobic scope does not predict the performance of a tropical eurythermal fish at elevated temperatures. *J. Exp. Biol.* **217**, 244–251.

Norin, T., Malte, H. and Clark, T. D. (2016). Differential plasticity of metabolic rate phenotypes in a tropical fish facing environmental change. *Funct. Ecol.* 369–378.

Nowak, B. (2001). Qualitative evaluation of risk factors for amoebic gill disease in cultured Atlantic salmon. In *Risk analysis in aquatic animal health* (ed. Rodgers, C. J.), pp. 148–154. Paris, France.

Nowak, B. F. (2012). Neoparamoeba perurans. In *Fish parasites: pathology and protection* (ed. Woo, P. T. K. and Buchmann, K.), pp. 1–18. CABI.

Nowak, B. F. and Munday, B. L. (1994). Histology of gills of Atlantic salmon during the first few months following transfer to sea water. *B. Eur. Assoc. Fish Pat.* **14**, 77–81.

O'Flynn, F. (1997). Comparisons of cultured triploid and diploid Atlantic salmon (*Salmo salar* L.). *ICES J. Mar. Sci.* **54**, 1160–1165.

Ojolick, E. J., Cusack, R., Benfey, T. J. and Kerr, S. R. (1995). Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture* **131**, 177–187.

Oliva-Teles, A. and Kaushik, S. J. (1990). Growth and nutrient utilization by 0 + and 1 + triploid rainbow trout, *Oncorhynchus mykiss*. *J. Fish Biol.* **37**, 125–133.

Oliver, E. C. J., Benthuysen, J. A., Bindoff, N. L., Hobday, A. J., Holbrook, N. J., Mundy, C. N. and Perkins-Kirkpatrick, S. E. (2017). The unprecedented 2015/16 Tasman Sea marine heatwave. *Nat. Commun.* **8**, 16101.

Ott, M. E., Heisler, N. and Ultsch, G. R. (1980). A re-evaluation of the relationship between temperature and the critical oxygen tension in freshwater fishes. *Comp. Biochem. Physiol. A: Physiol.* **67**, 337–340.

Ozolina, K., Shiels, H. A., Ollivier, H. and Claireaux, G. (2016). Intraspecific individual variation of temperature tolerance associated with oxygen demand in the European sea bass (*Dicentrarchus labrax*). *Conserv. Physiol.* **4**, cov060.

Pankhurst, N. W. and Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *J. Fish Biol.* **45**, 1013–1025.

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annu. Rev. Ecol. Evol. Syst.* **37**, 637–669.

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37–42.

Parsons, H. J., Nowak, B. F., Powell, M. D. and Dix, T. (2001). Changes in water quality during commercial freshwater bathing of Atlantic salmon (*Salmo salar*) in Tasmania - implications for treatment of amoebic gill disease. *B. Eur. Assoc Fish Pat.* **21**, 71–76.

Payne, N. L. and Smith, J. A. (2017). An alternative explanation for global trends in thermal tolerance. *Ecol. Lett.* **20**, 70–77.

Pedersen, B. H. (1997). The cost of growth in young fish larvae, a review of new hypotheses. *Aquaculture* **155**, 259–269.

Pennacchi, Y., Leef, M. J., Crosbie, P. B. B., Nowak, B. F. and Bridle, A. R. (2014). Evidence of immune and inflammatory processes in the gills of AGD-affected Atlantic salmon, *Salmo salar* L. *Fish Shellfish Immunol.* **36**, 563–570.

Pennacchi, Y., Adams, M. B., Nowak, B. F. and Bridle, A. R. (2016). Immune gene expression in the gills of Atlantic salmon (*Salmo salar* L.) following experimental reinfection with' ' *Neoparamoeba perurans*. *Aquaculture* **464**, 410–419.

Pennell, W. and McLean, W. E. (1996). Early Rearing. In *Principles of Salmonid Culture*, pp. 365–465. Elsevier.

Penney, C. M., Nash, G. W., Gamperl, A. K. and Tierney, K. (2014). Cardiorespiratory responses of seawater-acclimated adult Arctic char (Salvelinus alpinus) and Atlantic salmon (Salmo salar) to an acute temperature increase. *Can. J. Fish. Aquat. Sci.* **71**, 1096–1105.

Perry, A. L., Low, P. J., Ellis, J. R. and Reynolds, J. D. (2005). Climate change and distribution shifts in marine fishes. *Science* **308**, 1912–1915.

Peyghan, R. A. H. I. M. and Powell, M. D. (2006). Histopathological study of gills in experimentally amoebic gill disease (AGD) infected' ' Atlantic salmon, *Salmo salar*, L. *Iran. J. Vet. Res.* **7**, 8–13.

Pinheiro, J., Bates, D., DebRoy, S. and Sarkar, D. (2016). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-137, https://CRAN.R-project.org/package=nlme.

Popova, E., Yool, A., Byfield, V., Cochrane, K., Coward, A. C., Salim, S. S., Gasalla, M. A., Henson, S. A., Hobday, A. J., Pecl, G. T., et al. (2016). From global to regional and back again: common climate stressors of marine ecosystems relevant for adaptation across five ocean warming hotspots. *Glob. Chang. Biol.* **22**, 2038–2053.

Poppe, T. T., Johansen, R. and Torud, B. (2002). Cardiac abnormality with associated hernia in farmed rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.* **50**, 153–155.

Poppe, T. T., Johansen, R., Gunnes, G. and Tørud, B. (2003). Heart morphology in wild and farmed Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.* **57**, 103–108.

Pörtner, H. O. and Farrell, A. P. (2008). Ecology. Physiology and climate change. *Science* **322**, 690–692.

Pörtner, H. O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science* **315**, 95–97.

Pörtner, H. O., Berdal, B., Blust, R., Brix, O., Colosimo, A., De Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R., et al. (2001). Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). *Cont. Shelf Res.* **21**, 1975–1997.

Pörtner, H., Bock, C., Knust, R., Lannig, G., Lucassen, M., Mark, F. C. and Sartoris, F. J. (2008). Cod and climate in a latitudinal cline: physiological analyses of climate effects in marine fishes. *Clim. Res.* **37**, 253–270.

Powell, M. (2006). Physiological and clinical pathology. In *Systemic Pathology of Fish* (ed. Ferguson, H. W.), pp. 336–355. London: Scotian Press.

Powell, M. D. and Clark, G. A. (2003). In vitro survival and the effect of water chemistry and oxidative chemical treatments on isolated gill amoebae from AGD-affected Atlantic salmon. *Aquaculture* **220**, 135–144.

Powell, M. D. and Clark, G. A. (2004). Efficacy and toxicity of oxidative disinfectants for the removal of gill amoebae from the gills of amoebic gill disease affected Atlantic salmon (*Salmo salar* L.) in freshwater. *Aquac. Res.* **35**, 112–123.

Powell, M. D. and Nowak, B. F. (2003). Acid-base and respiratory effects of confinement in Atlantic salmon affected with amoebic gill disease. *J. Fish Biol.* **62**, 51–63.

Powell, M. D. and Perry, S. F. (1996). Respiratory and acid-base disturbances in rainbow trout (*Oncorhynchus mykiss*) blood during exposure to chloramine T, paratoluenesulphonamide, and hypochlorite. *Can. J. Fish. Aquat. Sci.* **53**, 701–708.

Powell, M. D. and Perry, S. F. (1997). Respiratory and acid-base disturbances in rainbow trout blood during exposure to chloramine-T under hypoxia and hyperoxia. *J. Fish Biol.* **50**, 418–428.

Powell, M. D. and Perry, S. F. (1999). Cardio-respiratory effects of chloramine-T exposure in rainbow trout. *J. Exp. Biol. online* **4**, 47–59.

Powell, M. D., Fisk, D. and Nowak, B. F. (2000). Effects of graded hypoxia on Atlantic salmon infected with amoebic gill disease. *J. Fish Biol.* **57**, 1047–1057.

Powell, M. D., Parsons, H. J. and Nowak, B. F. (2001). Physiological effects of freshwater bathing of Atlantic salmon (*Salmo salar*) as a treatment for amoebic gill disease. *Aquaculture* **199**, 259–266.

Powell, M. D., Forster, M. E. and Nowak, B. F. (2002a). Apparent vascular hypertension associated with amoebic gill disease affected Atlantic salmon (*Salmo salar*) in Tasmania. *B. Eur. Assoc. Fish Pat.* **22**, 328–333.

Powell, M. D., Nowak, B. F. and Adams, M. B. (2002b). Cardiac morphology in relation to amoebic gill disease history in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **25**, 209–215.

Powell, M., Attard, M. G., Harris, J. O., Roberts, S. D. and Leef, M. J. (2005). Why fish die-the treatment and pathophysiology of AGD. *FRDC project* (2001/205).

Powell, M. D., Leef, M. J., Roberts, S. D. and Jones, M. A. (2008). Neoparamoebic gill infections: host response and physiology in salmonids. *J. Fish Biol.* **73**, 2161–2183.

Quillet, E. and Gaignon, J. L. (1990). Thermal induction of gynogenesis and triploidy in Atlantic salmon (*Salmo salar*) and their potential interest for aquaculture. *Aquaculture* **89**, 351–364.

Rao, G. M. M. (1968). Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool.* **46**, 781–786.

Ridgway, K. R. (2007). Long-term trend and decadal variability of the southward penetration of the East Australian Current. *Geophys. Res. Lett.* **34**.

Roberts, S. D. and Powell, M. D. (2003a). Comparative ionic flux and gill mucous cell histochemistry: effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. A: Mol. Integr. Physiol. **134**, 525–537.

Roberts, S. D. and Powell, M. D. (2003b). Reduced total hardness of fresh water enhances the efficacy of bathing as a treatment for amoebic gill disease in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **26**, 591–599.

Roberts, S. D. and Powell, M. D. (2005). The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. *J. Comp. Physiol. B: Biochem. Syst. Environ. Physiol.* **175**, 1–11.

Robertson, L., Thomas, P., Arnold, C. R. and Trant, J. M. (1987). Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak. *The Progressive Fish-Culturist* **49**, 1–12.

Roche, D. G., Binning, S. A., Bosiger, Y., Johansen, J. L. and Rummer, J. L. (2013). Finding the best estimates of metabolic rates in a coral reef fish. *J. Exp. Biol.* **216**, 2103–2110.

Rodger, H. D. and McArdle, J. F. (1996). An outbreak of amoebic gill disease in Ireland. *Vet. Rec.* **139**, 348–349.

Rombough, P. J. and Ure, D. (1991). Partitioning of oxygen uptake between cutaneous and branchial surfaces in larval and young juvenile chinook salmon *Oncorhynchus tshawytscha*. *Physiol. Zool.* **64**, 717–727.

Roubal, F. R., Lester, R. J. G. and Foster, C. K. (1989). Studies on cultured and gill-attached *Paramoeba sp*. (Gymnamoebae: Paramoebidae) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *J. Fish Dis.* **12**, 481–492.

Sadler, J., Wells, R. M. ., Pankhurst, P. M. and Pankhurst, N. W. (2000). Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. *Aquaculture* **184**, 349–361.

Sadler, J., Pankhurst, P. M. and King, H. R. (2001). High prevalence of skeletal deformity and reduced gill surface area in triploid Atlantic salmon (*Salmo salar* L.). *Aquaculture* **198**, 369–386.

Sambraus, F., Olsen, R. E., Remen, M., Hansen, T. J., Torgersen, T. and Fjelldal, P. G. (2017). Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar*' ' L.) post-smolts. *Aquaculture* **473**, 1–12.

Sanchez-Quintana, D., Garcia-Martinez, V., Climent, V. and Hurle, J. M. (1995). Morphological analysis of the fish heart ventricle: myocardial and connective tissue architecture in teleost species. *Ann. Anat.* **177**, 267–274.

Sandblom, E., Clark, T. D., Gräns, A., Ekström, A., Brijs, J., Sundström, L. F., Odelström, A., Adill, A., Aho, T. and Jutfelt, F. (2016). Physiological constraints to climate warming in fish follow principles of plastic floors and concrete ceilings. *Nat. Commun.* **7**, 11447.

Sande, R. D. and Poppe, T. T. (1995). Diagnostic ultrasound examination and echocardiography in Atlantic salmon (*Salmo salar*). *Vet. Radiol. Ultrasound* **36**, 551–558.

Scarabello, M., Heigenhauser, G. J. and Wood, C. M. (1991). The oxygen debt hypothesis in juvenile rainbow trout after exhaustive exercise. *Respir. Physiol.* **84**, 245–259.

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856–1866.

Schurmann, H. and Steffensen, J. F. (1997). Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. *J. Fish Biol.* **50**, 1166–1180.

Scott, M. A. (2012). Performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges. PhD Thesis. University of British Columbia.

Scott, M. A., Dhillon, R. S., Schulte, P. M., Richards, J. G. and Magnan, P. (2015). Physiology and performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges. *Can. J. Fish. Aquat. Sci.* **72**, 125–134.

Shephard, K. L. (1994). Functions for fish mucus. *Rev. Fish Biol. Fish.* **4**, 401–429.

Shrimpton, J. M., Heath, J. W., Devlin, R. H. and Heath, D. D. (2012). Effect of triploidy on growth and ionoregulatory performance in ocean-type Chinook salmon: A quantitative genetics approach. *Aquaculture* **362-363**, 248–254.

Small, S. A. and Benfey, T. J. (1987). Cell size in triploid salmon. *J. Exp. Zool.* **241**, 339–342.

Snieszko, S. F. (1974). The effects of environmental stress on outbreaks of infectious diseases of fishes. *J. Fish Biol.* **6**, 197–208.

Soivio, A. and Tuurala, H. (1981). Structural and circulatory responses to hypoxia in the secondary lamellae ofSalmo gairdneri gills at two temperatures. *J. Comp. Physiol. B* **145**, 37–43.

Stehfest, K. M., Carter, C. G., McAllister, J. D., Ross, J. D., Semmens, J. M. (2017). Response of Atlantic salmon *Salmo salar* to temperature and dissolved oxygen extremes established using animal-borne environmental sensors. *Sci. Rep.* **7**, 4545.

Steinum, T., Kvellestad, A., Rønneberg, L. B., Nilsen, H., Asheim, A., Fjell, K., Nygård, S. M. R., Olsen, A. B. and Dale, O. B. (2008). First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L., and phylogeny of the causative amoeba using 18S cDNA sequences. *J. Fish Dis.* **31**, 205–214.

Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. Biol. Sci.* **278**, 1823–1830.

Sunday, J., Bates, A. and Dulvy, N. (2012). Thermal tolerance and the global redistribution of animals. *Nat. Clim. Chang*.

Swarup, H. (1959). Effect of triploidy on the body size, general organization and cellular structure in *Gasterosteus aculeatus* (L). *J. Genet.* **56**, 143–155.

Takahashi, S., Nakamura, E. and Okabe, S. Effects of cytokines, without and with *Helicobacter pylori* components, on mucus secretion by cultured gastric epithelial cells. *Dig. Dis. Sci*. **43**, 2301-2308.

Taylor, R. S., Muller, W. J., Cook, M. T., Kube, P. D. and Elliott, N. G. (2009a). Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. *Aquaculture* **290**, 1–8.

Taylor, R. S., Kube, P. D., Muller, W. J. and Elliott, N. G. (2009b). Genetic variation of gross gill pathology and survival of Atlantic salmon (*Salmo salar* L.) during natural amoebic gill disease challenge. *Aquaculture* **294**, 172–179.

Terblanche, J. S., Hoffmann, A. A., Mitchell, K. A., Rako, L., le Roux, P. C. and Chown, S. L. (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *J. Exp. Biol*. **214**, 3713-3725.

Teskeredžić, E., Donaldson, E. M., Teskeredžić, Z., Solar, I. I. and McLean, E. (1993). Comparison of hydrostatic pressure and thermal shocks to induce triploidy in coho salmon (*Oncorhynchus kisutch*). *Aquaculture* **117**, 47–55.

Tewksbury, J. J., Huey, R. B. and Deutsch, C. A. (2008). Ecology. Putting the heat on tropical animals. *Science* **320**, 1296–1297.

Thorarensen, H., Gallaugher, P. E., Kiessling, A. K. and Farrell, A. P. (1993). Intestinal blood flow in swimming Chinook salmon, Oncorhynchus tshawytscha, and the effects of haematocrit on blood flow distribution. *J. Exp. Biol*. **179**, 115-129.

Thorburn, M. A. and Moccia, R. D. (1993). Use of chemotherapeuties on trout farms in Ontario. *J. Aquat. Anim. Health* **5**, 85–91.

Toepfer, C. and Barton, M. (1992). Influence of salinity on the rates of oxygen consumption in two species of freshwater fishes, Phoxinus erythrogaster (family Cyprinidae) and Fundulus catenatus (family Fundulidae). *Hydrobiologia*. **242**, 149-154.

Tota, B. and Gattuso, A. (1996). Heart ventricle pumps in teleosts and elasmobranchs: A morphodynamic approach. *J. Exp. Zool.* **275**, 162-171.

Tyndall, J. (1863). XXVII. On radiation through the earth's atmosphere. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science* **25**, 200–206.

Van Leeuwen, T. E., Rosenfeld, J. S. and Richards, J. G. (2012). Effects of food ration on SMR: influence of food consumption on individual variation in metabolic rate in juvenile coho salmon (*Onchorhynchus kisutch*). *J. Anim. Ecol.* **81**, 395–402.

Verdugo, P. (1984). Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: a new model. In *Mucus and mucosa*, 212–222.

Verhille, C., Anttila, K. and Farrell, A. P. (2013). A heart to heart on temperature: impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **164**, 653–657.

Wallach, M. and Nowak, B. F. (2012). Understanding amoebic gill disease. *An. Sci. Rev. 2011* p. 199.

Wedemeyer, G. A., Barton, B. A. and Mcleay, D. J. (1990). Stress and Acclimation. In *Methods for fish biology*, pp. 451–489. Bethesda, Maryland.

Wells, P. and Pinder, A. (1996). The respiratory development of Atlantic salmon. II. Partitioning of oxygen uptake among gills, yolk sac and body surfaces. *J. Exp. Biol.* **199**, 2737–2744.

Wetzel, R. G. (2001). *Limnology: Lake and River Ecosystems*. illustrated. Gulf Professional Publishing.

Withler, R. E., Beacham, T. D., Solar, I. I. and Donaldson, E. M. (1995). Freshwater growth, smolting, and marine survival and growth of diploid and triploid coho salmon (*Oncorhynchus kisutch*). *Aquaculture* **136**, 91–107.

Wong, F. Y. K., Carson, J. and Elliott, N. G. (2004). 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. *Dis. Aquat. Org.* **60**, 65–76.

Wood, C. (1991). Acid-base and ion balance, metabolism, and their interactions, after exhaustive exercise in fish. *J. Exp. Biol.* **160**, 285–308.

Wood, C. M. and McDonald, D. G. (1997). *Global warming: implications for freshwater and marine fish*. (ed. Cambridge University Press).

Wood, C. M. and Perry, S. F. (1985). Respiratory, circulatory, and metabolic adjustments to exercise in fish. In *Circulation, Respiration, and Metabolism* (ed. Gilles, R.), pp. 2–22. Berlin, Heidelberg: Springer Berlin Heidelberg.

Yamamoto, A. and Iida, T. (1994). Oxygen consumption and hypoxic tolerance of triploid rainbow trout (*Oncorhynchus mykiss*). *Fish Pathol. (Japan)*.

Yamamoto, T., Ueda, H. and Higashi, S. (1998). Correlation among dominance status, metabolic rate and otolith size in masu salmon. *J. Fish Biol.* **52**, 281–290.

Young, N. D., Crosbie, P. B. B., Adams, M. B., Nowak, B. F. and Morrison, R. N. (2007). *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). *Int. J. Parasitol.* **37**, 1469–1481.

Yousaf, M. N., Koppang, E. O., Skjødt, K., Hordvik, I., Zou, J., Secombes, C. and Powell, M. D. (2013). Comparative cardiac pathological changes of Atlantic salmon (*Salmo salar* L.) affected with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD). *Vet. Immunol. Immunopathol.* **151**, 49–62.

Zilberg, D. and Munday, B. L. (2000). Pathology of experimental amoebic gill disease in Atlantic salmon, Salmo salar L., and the effect of pre-maintenance of fish in sea water on the infection. *J. Fish Dis.* **23**, 401–407.

Zuchelkowski, E. M., Pinkstaff, C. A. and Hinton, D. E. (1985). Mucosubstance histochemistry in control and acid-stressed epidermis of brown bullhead catfish, *Ictalurus nebulosus* (LeSueur). *Anat. Rec.* **212**, 327–35, 396.

References